# Characterisation of allergens in pilchard, responsible for the development of occupational allergy in the seafood processing industry in the Western Cape

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# Declaration

I, Christoff Willem Hikuam, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed..... Date.....

# Abstract

The increase in popularity of fish, coupled with technological advances in the fishing industry and changes in the control and management of fishing resources, has led to a significant increase of fish processing workers from 13 million in 1970 to 38 million in 2002. Whereas allergic reactions to fish proteins were previously only documented in consumers, increasing reports of occupational fish allergies of fish processing workers has become evident. In South Africa, the reported prevalence of occupational asthma associated with fish processing workers is 2 – 8%, and the prevalence of occupational protein contact dermatitis 3 – 11%. Pilchard is one of the most consumed fish species in South Africa and the immunological analysis of this species will therefore contribute to the provision of occupational health services in the pilchard processing industry.

Proteins extracted from fresh, frozen and canned pilchard (*Sardinops sagax*), as well as fresh samples of six other processed and consumed South African fish were characterised by denaturing protein electrophoresis and immunoblotted with different monoclonal and polyclonal antibodies. Sera from sensitised workers were subsequently used to characterise the membrane-bound pilchard proteins and analysed for human Immunoglobulin G (IgG) and Immunoglobulin E (IgE) antibodies to determine antigen recognition.

A protein of 12 kDa molecular weight was found to be present in all fish protein extracts, however, at various concentrations. With the aid of the monoclonal and

polyclonal antibodies, the 12 kDa protein was postulated to be parvalbumin, a known allergen in some fish species. Immunoblotting experiments for the identification of workers' IgG- and IgE-reactivities to fresh, frozen and canned pilchard showed a 12 kDa protein as an immunolgically reactive fish protein. This protein was also found to occur in dimeric, trimeric and tetrameric forms, which may have significant implications in the diagnosis and management of occupational sensitisation to pilchard.

# Dedication

To my wife, Felicita. All my love and respect to you.

# Acknowledgements

My appreciation goes out to:

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# Abbreviations

| | |
|---|---|
| AMPS | Ammonium Persulphate |
| AP | Alkaline Phosphatase |
| APC | Antigen Presenting Cells |
| BCA | Bicinchoninic Acid |
| BCIP | 5-Bromo-4-Chloro-3-Indolyl Phosphate |
| BHR | Basophil Histamine Release |
| $Ca^{2+}$ | Calcium Ions |
| cDNA | Complementary Deoxyribonucleic Acid |
| DBPCFC | Double Blind Placebo-Controlled Food Challenge |
| °C | Degrees Celsius |
| $dH_2O$ | Distilled Water |
| DNA | Deoxyribonucleic Acid |
| EKG | Electrocardiogram |
| ELISA | Enzyme-linked Immunosorbent Assay |
| Fc | Crystallisable Region of Antibody |
| g | Gravities |
| HPLC | High Performance Liquid Chromatography |
| IgE | Immunoglobulin E |
| IL | Interleukin |
| kDa | Kilo Dalton |
| km | Kilometre |
| kUA | Kilo Units Antigen Per Litre |

| | |
|---|---|
| L | Litre |
| M | Molar |
| µg | Microgram |
| µl | Microlitre |
| mA | Milli Ampere |
| mg | Milligram |
| ml | Millilitre |
| mm | Millimetre |
| Mw | Molecular Weight |
| NBT | Nitro Blue Tetrazolium |
| nm | Nanometre |
| pI | Isoelectric Point |
| PBS | Phosphate Buffered Saline |
| PVDF | Polyvinylidene Difluoride |
| RAST | Radio Allergosorbent Test |
| rpm | Revolutions Per Minute |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| SPT | Skin Prick Test |
| TBS | Tris Buffered Saline |
| TEMED | N, N, N', N',-Tetramethyl-ethylenediamine |
| TH | T Helper |
| V | Volt |
| w/v | Weight Per Volume |

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# Chapter 1

# Introduction

## 1.1 General Introduction

Diets containing fish are increasingly recommended by physicians because of its importance as a source of highly digestible protein, polyunsaturated fatty acids and fat-soluble vitamins (Bernhisel-Broadbent *et al.*, 1992). In 1995, developed countries accounted for 85 percent of total fish imports, due to the increased popularity of fish and other seafood in wealthier countries, coupled with improvements in preserving, processing, and transporting the catch from the developing world (Food and Agricultural Organisation. The state of world fisheries and aquaculture. Rome: FAO, 1997).

The fishing industry has undergone considerable change due to technological advances as well as changes in the control and management of fishing resources. From a survey that was carried out by the Food and Agricultural Organisation of the United Nations in 2004, it was estimated that between 1970 and 2002, the number of people involved in all fishing activities increase from approximately 13 million to 38 million.

In China, where the combined numbers of fishers and fish farmers (12.3 million) represent nearly one-third of the world total, in 2002, 8.4 million people worked in

capture fisheries and 3.9 million in aquaculture (Food and Agricultural Organisation. 2004. The state of world fisheries and aquaculture, Part 1: World review of fisheries and aquaculture: FAO).

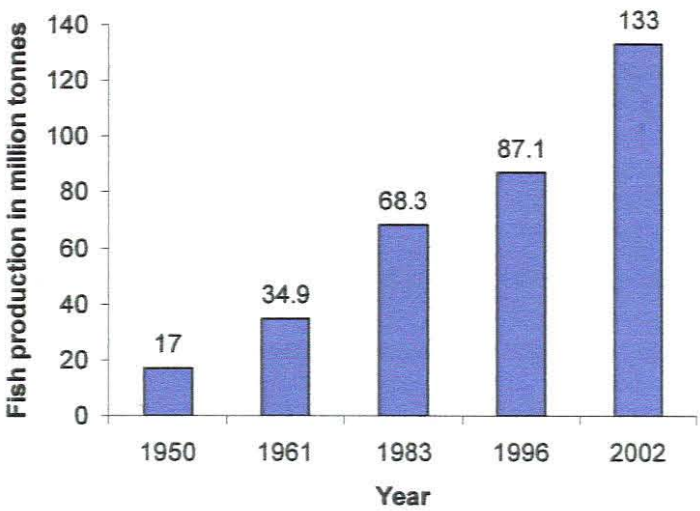


**Figure 1. Growth in world capture of fish.** The capture of fish increased more than 7 times in 52 years. (Modified from ILO. Safety and health in the fishing industry. Geneva: ILO, 1999; Food and Agricultural Organisation. 2004. The state of world fisheries and aquaculture, Part 1: World review of fisheries and aquaculture: FAO).

South Africa has a coastline extending 2798 km, with a highly productive western coastline, and an eastern coastline which is less productive, although it has higher species diversity. Fisheries are still a relatively small sector within the national economy of South Africa, with an overall contribution of about one percent to the national gross domestic product. The Western Cape, with areas such as Saldanha Bay and St. Helena Bay, is the centre of industrial fisheries in South Africa, with Cape Town, Mossel Bay and Port Elizabeth being other major centres of fisheries-related employment and income generation. Apart from the main industrial fisheries, recreational line fishery contributes significantly to the economy, but this contribution

is difficult to quantify (Food and Agriculture Organisation. Fishery Country Profile: The Republic of South Africa: FAO, 2005).

The fishing industry in South Africa employs approximately 30 000 workers directly and 60 000 more workers in related jobs, supplying the entire Southern African region. In 1999, the total harvest of seafood in South Africa amounted to 571 924 tonnes, with bony fish, hake and pilchard as the most commonly processed seafoods (Jeebhay *et al.*, 2000).

Previously, allergies to seafood have been documented mainly among consumers (Lehrer, 1990), but with the increase in seafood production, its processing and distribution has increased, leading to more frequent reporting of occupational seafood allergies among fish processing workers. In South Africa, the reported prevalence of occupational asthma associated with fish processing workers is 2-8%, and the reported prevalence of occupational protein contact dermatitis is 3-11% (Jeebhay *et al.*, 2001). In a South African study reported in 2000, none of the seafood processing workplaces assessed had industrial hygiene programs for seafood aerosol exposure in place, while about 50% provided an occupational health service and medical surveillance of workers. A positive trend however was observed between the size of the workforce and the provision of occupational health services (Jeebhay *et al.*, 2000). Furthermore, another study reported in 2004 compared the findings from South African seafood industries with ones obtained in Australia. Eighteen percent of 140 workplaces responded to the cross-sectional employer-based survey in Australia. From this group, 9% of the workplaces had industrial hygiene programs for seafood aerosols, with about half of the total participating workplaces providing an

occupational health service and medical surveillance of workers. Both in South Africa and Australia, skin rash accounted for 78-81% of all reported health problems, followed by asthmatic symptoms (7-10%), and other non-specific allergic symptoms (9-15%). From this study it was concluded that there were great similarities between the two countries concerning the significantly elevated prevalence of work-related allergies (Lopata *et al.*, 2004).

## 1.2 Aims and Objectives

Although the first major allergen in fish was isolated and characterised as early as 1969 by Aas and Elsayed, allergens of pilchard have not been isolated or characterised before (Aas and Elsayed, 1969a) In this study it was aimed to identify the major allergen(s) of pilchard and compare it to previously characterised allergens from other fish species.

The first objective was to prepare protein extracts from the following commercially important fish species: Fresh, frozen and canned pilchard (*Sardinops sagax*); tuna (*Thunnus albacares)*; hake (*Merluccius* spp); snoek (*Thyrsites atun)*; yellow tail (*Seriola lalandi*), Alaska pollack (*Theragra chalcogramma)*; and anchovy (*Engraulis capensis*). Biochemical analysis was then performed by total protein concentration determinations and denaturing gel electrophoresis to establish the molecular size and distribution of the individual proteins in the fish extracts.

Secondly, an immunological assessment of the reactivity of these extracts with a monoclonal anti-parvalbumin antibody as well as polyclonal rabbit anti-pilchard and rabbit anti-anchovy antibodies had to be carried out to characterise antigenic proteins.

Thirdly, the Immunoglobulin G- and Immunoglobulin E-binding to fresh, frozen, and canned pilchard with sera from sensitised seafood processing workers had to be established and reactivity profiles analysed in order to identify the major and minor allergen(s) in pilchard.

# Chapter 2

# Literature Review

## 2.1 Classification of Seafood

Edible seafood comprise of three major divisions of sea organisms, which are grouped as the Chordata, the Mollusca, and the Arthropoda. The major class in the Chordata is the Osteichthyes or ray-fin fishes, while snails and abalone (Gastropods), mussels, oysters, scallops and clams (Bivalves), as well as squid and octopus (Cephalopods) are all included in the Mollusca. Crustacea is the major class of the Anthropoda, and they include shrimp, crabs, lobsters and crawfish (Lehrer *et al.*, 2003).

According to Nelson (1994), there are 482 fish families distributed in lakes, streams, estuaries, and oceans worldwide, containing 24 618 species in total. About 40% of these species live in fresh water. Chordates belong to the superphylum Deuterostomia, together with phyla such as Chaetognatha, Hemichordata and Echinodermata. The Chordates used for the purpose of the experiments in this study are illustrated in Figures 2.1 to 2.7.

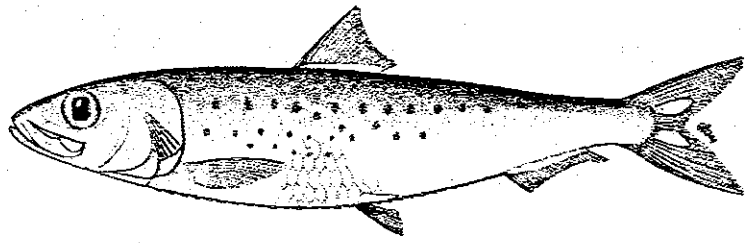

**Figure 2.1. An illustration of pilchard** (*Sardinops sagax*). Source: www.fishbase.org.

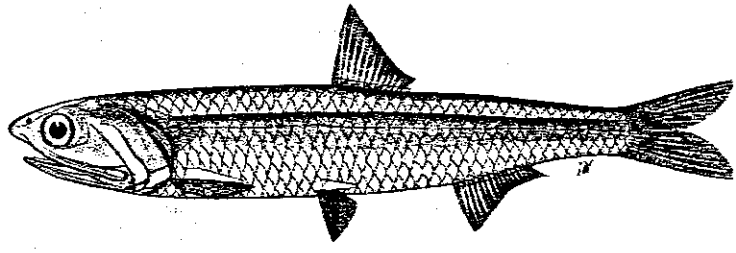

**Figure 2.2. An illustration of Cape anchovy** (*Engraulis capensis*). Source: www.fishbase.org.

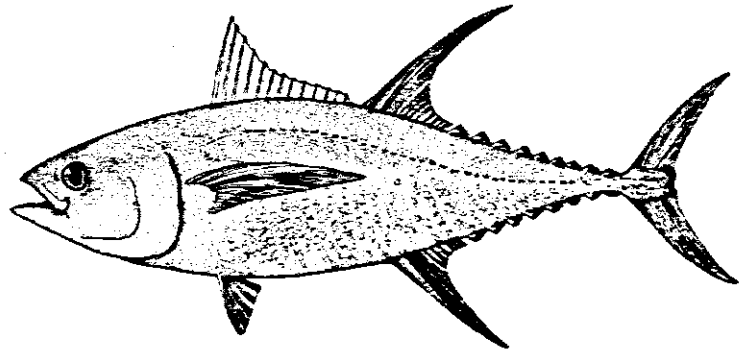

**Figure 2.3. An illustration of yellow fin tuna** (*Thunnus albacares)*. Source: www.fishbase.org.

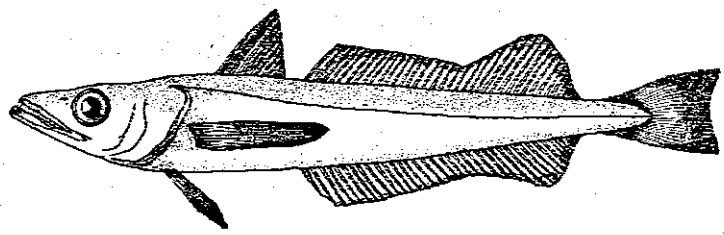

**Figure 2.4. An illustration of Cape hake** (*Merluccius* spp). Source: www.fishbase.org.

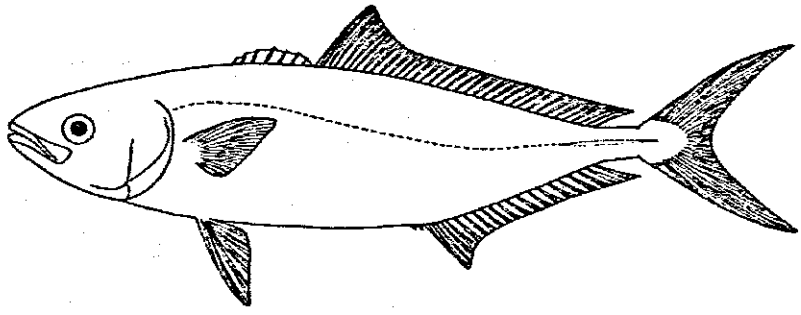

**Figure 2.5. An illustration of Cape yellow tail** (*Seriola Lalandi*).Source: www.fishbase.org.

**Figure 2.6. An illustration of snoek** (*Thyrsites atun*). Source: www.fishbase.org.

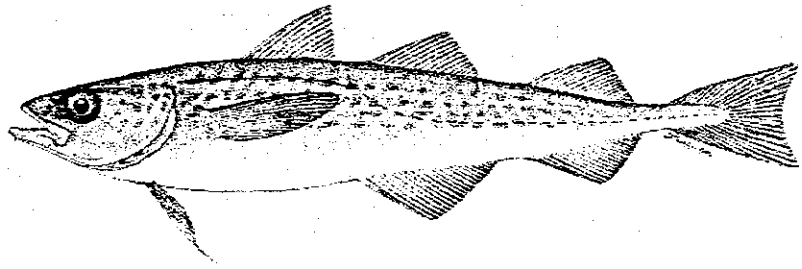

**Figure 2.7. An illustration of Alaska pollack** (*Theragra chalcogramma)*. Source: www.fishbase.org.

## 2.2 Food Allergy

Food allergy is defined as a hypersensitivity reaction to food, which can produce a range of symptoms affecting the skin, respiratory tract, and the gastrointestinal tract through IgE-mediated and non IgE-mediated reactions (Sampson, 2003). Disorders with acute onset of symptoms after ingestion are usually IgE-mediated. In this case, the tissue mast cells and blood basophils become sensitised and upon re-exposure the causal food proteins bind to the specific IgE molecules and trigger the release of mediators, such as histamine, that cause allergic symptoms. Subacute or chronic food hypersensitivity disorders, which are mediated mainly by T-cells, also exist. Chronic disorders, attributed to food allergy with measurable IgE, have also been observed (Sampson and Anderson, 2000). Johansson *et al* (2001) revised the nomenclature of allergy in 2001, and classified food hypersensitivity as shown in Figure 2.8.

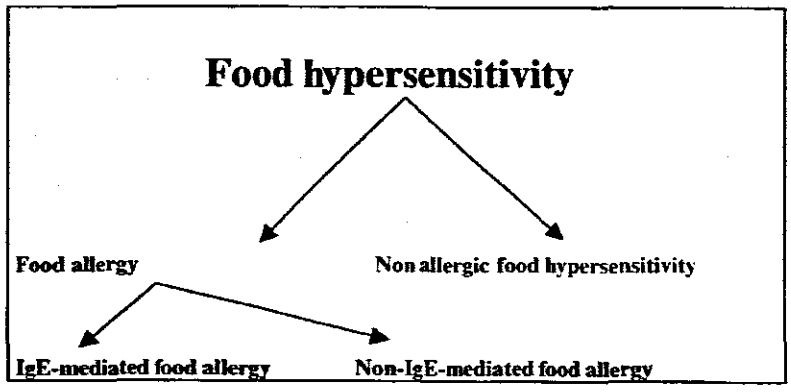


**Figure 2.8. Classification of food hypersensitivity.** Food hypersensitivity can either be a food allergy, or a non-allergic food hypersensitivity. Food allergy is further sub-divided into IgE- or non IgE-mediated food allergy (Johansson *et al.*, 2001).

## 2.3 IgE in Allergic Response

The foundations of the discovery of IgE started as early as 1921, when Prausnitz and Kustner executed a classical experiment, in which Prausnitz passively sensitised the skin in his forearm to fish by injecting fish-allergic serum from Kustner, who was his co-worker. The following day, after injecting himself with a fish extract at the same site in the skin, Prausnitz developed an immediate-type wheal and flare reaction. At that time, the result of the experiment was called the P-K reaction, and ultimately indicated the existence of the "reagin" or serum factor that could spontaneously sensitise skin and mediate positive immediate-type skin reactions (Prausnitz and Kustner, 1921). Further intensive studies over 40 years led to the term Immunoglobulin E (IgE) being accepted as that component in serum that is associated with allergenic activity (Bennich, 1968).

IgE is known to be synthesised by a very small proportion of plasma cells in the body, therefore only very low concentrations are usually present in serum. When allergens

enter the body, Antigen Presenting Cells (APCs) at the site of entry recognises, processes, and presents them to T helper (TH) cells. The TH2 cells then secrete Interleukin-4 (IL-4) and IL-10, which trigger the B cells to produce and secrete IgE as depicted in Figure 2.9. IgE binds to tissue mast cells and blood basophils with high affinity through its Fc portion, thus sensitising them. When the allergen reaches the sensitised mast cells, it cross-links the surface-bound IgE, causing an increase in the intracellular calcium ($Ca^{2+}$), which in turn triggers the release of pre-formed mediators, such as histamine and proteases. IL-3 and IL-4, which are released as a result of IgE-mediated mast cell activation, also result in increased autocrine effects on the mast cell and production of more IgE. Therefore, IgE levels are often raised in allergic disease, with the presence of preformed and newly formed mediators, which are responsible for the pharmacological and clinical effects, discussed under the symptoms of food allergy (Section 2.6). This is called a Type I (immediate) hypersensitivity reaction (Roitt, *et al.*, 2001).

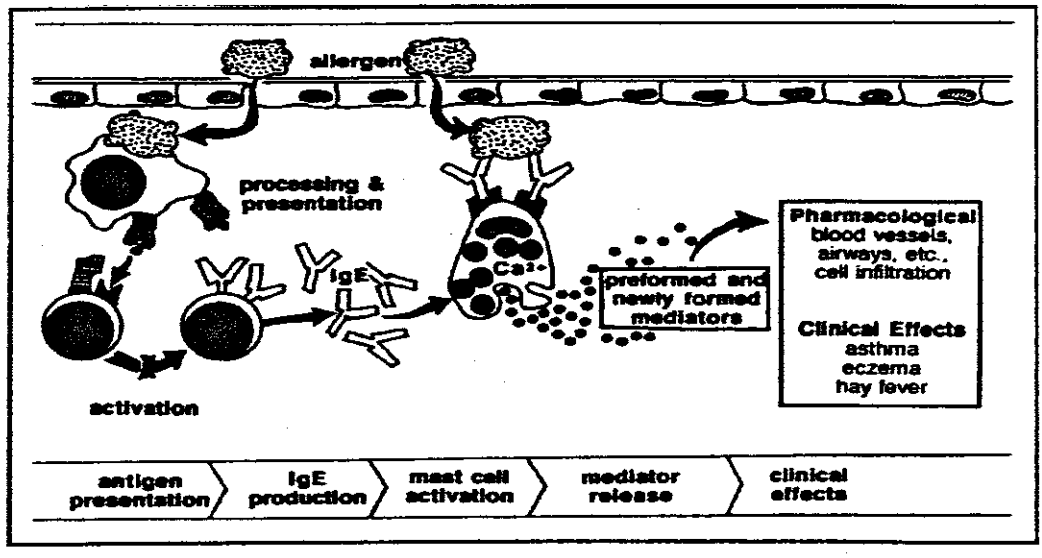


**Figure 2.9. The Allergic Reaction.** The cascade of events taking place at the site of allergen invasion across the mucosal membrane barriers with the resultant mast cell activation, mediator release, and clinical effects (Lehrer *et al.*, 2003).

## 2.4 Food Allergens

Since food allergy might either result from a breach in oral tolerance to ingested foods or during respiratory exposure, it can be divided into two classes. Class 1 food allergy is caused by food proteins which are generally stable to digestion, whilst in contrast, Class 2 food allergy is typically caused by labile proteins which are encountered through the respiratory route (Breiteneder and Ebner, 2003).

Class 1 food allergens are water-soluble glycoproteins, ranging between 10 and 70 kDa in molecular weight. These proteins are generally stable to heat, acid and proteases, therefore they are capable of inducing a sensitisation through the gastrointestinal route following ingestion (Breiteneder and Mills, 2005). Class 2 food allergens, on the other hand, are mostly profilins, which are heat-labile and prone to enzymatic breakdown, therefore will unlikely cause sensitisation through the gastrointestinal tract, but more readily through the respiratory route (Breiteneder and Radauer, 2004).

### 2.4.1 Major Fish Allergens

The isolation, purification, and characterisation of fish allergens date back to 1969, with Aas and Elsayed, characterising the major Baltic cod (*Gadus callarias*) allergen (Gad c 1), which was formerly known as Allergen M (Aas and Elsayed, 1969a). This parvalbumin protein was the first to be characterised and sequenced over 20 years ago. In 1975, Allergen M was known to be an acidic protein with a molecular weight of 15 kDa. It consisted of 114 amino acids and only one

carbohydrate residue. The allergen was further purified into two fragments, TM1 and TM2 (Elsayed and Bennich, 1975). TM1 had a molecular weight of 8.5 kDa, and TM2 a molecular weight of 6.5 kDa. These fragments have been shown to be equally active and allergenically identical in biological tests, but less active than Allergen M itself. As the first reported example of a full structural analysis of an active allergen, the amino acid sequence of TM2 was presented in this work (Aas and Elsayed, 1969b).

More than 20 years later, Lindstrom and co-workers purified a similar parvalbumin from the white muscle of Atlantic salmon (*Salmo salar*). The salmon parvalbumin was purified by anion exchange chromatography, gel filtration chromatography, and high-performance liquid chromatography (HPLC) of the muscle extracts. It was demonstrated to be antigenic, as well as allergenic by use of cod and salmon allergic patients' sera in addition to polyclonal cod and salmon antibodies in IgG Enzyme-liked immunosorbent assay (ELISA) and immunoelectrophoretic methods. This protein was named Sal s 1 and found to be the major allergen of Atlantic salmon, with a molecular weight which was calculated, based on its GenBank sequence, to be 11.9 kDa (Linstrom *et al.*, 1996).

Following that, parvalbumin in Carp (*Cyprinus carpio*) was purified, and characterised biochemically as well as immunologically in a study conducted by Bugajska-Schretter and colleagues in 2000. Using immunogold electron microscopy and cell fractionation, the tissue and subcellular distribution of carp parvalbumin was analysed. The parvalbumin fraction was also purified to homogeneity, using a diethylaminoethyl column, and further analysed by mass spectrometry and circular

dichroism spectrometry. Moreover, its allergenic activity was analysed by IgE binding of sera from patients allergic to fish and by means of basophil histamine release tests. Parvalbumin was subsequently detected in ultrathin sections of carp muscle fibres and fibre cells, but not in connective tissue sections. Using a monoclonal anti-parvalbumin antibody, subcellular section studies revealed parvalbumin reactivity in cytosolic fractions, whereas nuclear fractions showed no reactivity. Parvalbumin was detected as an abundant cytosolic carp muscle protein, using sera from fish-allergic patients. The purified IgE-reactive carp parvalbumin had an isoelectric point (pI) of 4.7, and contained most IgE epitopes present in natural fish extracts, having a mean IgE-binding inhibition of 81% to cod, 89% to tuna, and 78% to salmon extract. The purified carp parvalbumin also induced dose-dependent basophil histamine release from an individual with fish allergy, but not from an atopic individual (Bugajska-Schretter *et al.*, 2000).

Furthermore, Swoboda *et al* (2002) constructed a cDNA expression library from carp (*Cyprinus carpio*) muscle and used serum IgE from a fish-allergic patient to isolate two cDNAs coding for carp parvalbumin isoforms, Cyp c 1.01 and Cyp c 1.02. The Cyp c 1.01 was then over-expressed in *Escherichia coli*, and rCyp c 1.01 was purified to homogeneity from it. Circular dichroism analysis showed that rCyp c 1.01 represented a folded protein with mainly a α-helical secondary structure, and mass spectroscopy analysis indicated its molecular weight to be 11.4 kDa. Subsequently, immunoblots and inhibition experiments showed that rCyp c 1.01 was recognised by IgE antibodies of all fish-allergic patients (n=60) who previously reacted to natural carp parvalbumin, and recombinant carp parvalbumin completely blocked IgE binding to the natural carp parvalbumin. Quantitative IgE inhibition experiments also showed

that rCyp c 1.01 contained 70% of IgE epitopes present in allergen extracts of various fish species. These results suggested the possibility of rCyp c 1.01 being used as a marker allergen to diagnose IgE-mediated cross-sensitisation to various fish species (Swoboda *et al.*, 2002).

Recently, a new parvalbumin allergen in Atlantic codfish (*Gadus morhua*) was identified by Das Dores *et al.* Initially, four proteins with respective molecular weights of 12.5, 24, 38, and 51 kDa were detected using an anti-parvalbumin antibody, indicating that they all belonged to the parvalbumin family. In the native form, parvalbumin Gad m 1 was present at 24 kDa in molecular size, but an 11.5 kDa molecular size was deduced from analysing the cDNA sequence. This indicated that the parvalbumin in Atlantic codfish was present in dimeric form, and since the Western blots with an anti-parvalbumin antibody after SDS-PAGE under reducing conditions also appeared at 38 and 51 kDa, the parvalbumin was present in trimeric and tetrameric forms as well. The sequence alignment of amino acids confirmed calcium binding sites, a cysteine in position 18, an arginine in position 75, and a quadruplet of acidic residues in the region 59-62 amino acids. These above-mentioned characteristics are typical of β-parvalbumins. In addition, Gad m 1 shares greater homology of amino acid sequence with Sal s 1 from salmon (75%) than with Gad c 1 from Baltic cod (62.3%) (Das Dores *et al.*, 2002).

In 2003, parvalbumin proteins Sco j 1, Sco a 1, and Sco s 1 (*Scomber japonis, S. australasicus, and S. scrombus*, respectively) were suggested to be the major allergens in mackerel by Hamada and colleagues, after purification from the white muscle of these three species by gel filtration on Sephadex G-75 and reverse-phase

HPLC. Four of five sera from fish allergic patients reacted to all the purified parvalbumins in ELISA experiments, demonstrating that parvalbumin is the major allergen in mackerel. ELISA inhibition experiments showed that these allergens have very similar IgE-binding epitopes and also show a high degree of cross-reactivity (Hamada *et al.*, 2003).

Similarly, in 2005, The c 1, the major allergen in Alaska pollack (*Theragra chalcogramma*) was characterised and compared with codfish Gad c 1. The c 1 was partially purified, revealing two distinct fractions of parvalbumin, which were demonstrated to have similarly high IgE- and IgG-binding capacities. These isoforms were designated P1 and P2 with isoelectric points of 4.39 and 4.60, respectively. It displayed a similar pattern as parvalbumin of cod in SDS-PAGE, and the immunoblotting with 6 sera from fish-allergic patients also showed similar IgE-binding of The c 1 and Gad c 1, but the concentration required for obtaining 50% inhibition of IgE-binding to cod parvalbumin was 18% higher for The c 1 than Gad c 1. The c 1 has a molecular weight of 11.5 kDa compared to the 12.3 kDa of Gad c 1 (Van Do *et al.*, 2005b). Table 2.1 summarises parvalbumin allergens in six different types of fish, which were previously isolated and characterised.

**Table 2.1 Summary of major fish allergens in six types of fish**

| Name | Origin | Isoelectric Point | Molecular weight in kDa | Amino Acid Homology (%) to Gad c 1 | Reference |
|---|---|---|---|---|---|
| Gad c 1, parvalbumin | Baltic cod (*Gadus callarias*) | 4.75 | 12.3 | N/A | Elsayed and Bennich, 1975 |
| Sal s 1, parvalbumin | Atlantic salmon (*Salmo salar*) | Not stated | 11.9 | Not stated | Linstrom *et al.*, 1996 |
| Cyp c 1.01, and Cyp c 1.02 parvalbumin | Carp (*Cyprinus carpio*) | 4.41 (Cyp c 1.01) and 4.77 (Cyp c 1.02) | 11.4 | 68% | Swoboda *et al.*, 2002 |
| Gad m 1, parvalbumin | Atlantic codfish (*Gadus morhua*) | 4.34 | 11.5 | 62.3% | Das Dores *et al.*, 2002 |
| Sco j 1, Sco a 1, and Sco s 1, respectively, all parvalbumin | Mackerel *Scomber japonis, S. australasicus, and S. scrombus,* | Not stated | +/-11.0 | 58% (Sco j 1) | Hamada *et al.*, 2003 |
| The c 1 (P 1 and P 2), parvalbumin | Alaska Pollack (*Theragra chalcogramma*) | 4.39 (P 1) and 4.60 (P 2) | 11.5 | 59% (P 1) and 62% (P 2) | Van Do *et al.*, 2005 |

### 2.4.2 Other Fish Allergens

The allergens of two highly consumed Indian fishes, hilsa (*Tenualosa ilisha*) and pomfret (*Pampus argenteus*) were identified, using ELISA with sera from fish-allergic patients. The visualisation of proteins in fish extracts by SDS-PAGE and immunoblot analysis, demonstrated IgE reactivity of patients. The analysis confirmed the SPT and ELISA results, which revealed that all the fish-allergic patients had increased levels of specific IgE and Skin Prick Test (SPT)-positivity.

Surprisingly, in this study, the patients' sera could not recognise any low molecular weight proteins, such as parvalbumin. Instead, a 50 kDa and some higher molecular weight proteins were revealed as common allergens in hilsa and pomfret, as summarised in Table 2.2 (Das *et al.*, 2005).

Collagen was identified as a fish allergen for the first time by Hamada *et al* in a study conducted in 2001. In this study, SDS-PAGE, immunoblotting, and amino acid analysis of the purified high molecular weight protein from bigeye tuna (*Thunnus obesus*) was used to demonstrate that the sera of 5 out of 8 subjects in the study were IgE reactive. The SDS gel, after staining with Coomassie Brilliant Blue R-250, as well as the immunoblot, with sera against an anti-human IgE antibody, detected IgE reactivity at 120 and 240 kDa. According to this group, these 2 bands appeared to be α- and β-chains of collagen, respectively. Collagen from heated extracts of three kinds of meat (beef, pork, and chicken), two species of crustaceans (tanner crab and giant tiger shrimp), and three species of molluscs (oyster, turban shell, and squid) showed no IgE reactivity to sera from three of the subjects tested, suggesting no

antigenic cross-reactivity between collagens from fish and that from other animals (Hamada *et al.*, 2001).

**Table 2.2 IgE-binding profile of proteins from hilsa and pomfret**
(Modified from Das *et al.*, 2005)

| **Hilsa** | **Molecular Weight in kDa** | | | | | |
|---|---|---|---|---|---|---|
| **Patients' sera** | 29 | 38 | 41 | 50 | 62 | 94 |
| 1 | X | X | X | X | X | - |
| 2 | X | X | X | X | X | - |
| 3 | X | X | X | X | X | - |
| 4 | - | X | X | X | X | - |
| 5 | - | - | X | X | X | - |
| 6 | - | - | X | X | X | - |
| 7 | X | X | X | X | X | X |
| 8 | X | X | X | X | X | X |
| 9 | X | - | X | X | X | X |
| 10 | - | - | X | X | X | X |
| **Pomfret** | **Molecular Weight in kDa** | | | | | |
| **Patients' sera** | 32 | 35 | 43 | 50 | 68 | 97 |
| 1 | X | - | X | - | X | X |
| 2 | X | - | X | - | X | X |
| 3 | X | X | X | X | - | - |
| 4 | X | X | - | X | - | X |
| 5 | X | X | X | X | - | X |
| 6 | X | X | X | X | - | X |
| 7 | X | X | - | X | - | X |
| 8 | X | X | X | X | - | X |
| 9 | X | X | X | X | X | X |
| 10 | X | X | - | X | - | X |

**Key:** X = presence of band

- = absence of band

### 2.4.3 Other Seafood Allergens

In a review by Lehrer *et al* in 2003, parvalbumin was acknowledged to be the major allergen in fish, while tropomyosin was generally accepted as the major allergen in other seafoods. Table 2.3 shows the major allergens in allergenic seafood, excluding fish.

**Table 2.3 Examples of major seafood allergens**
(Modified from Lehrer *et al.*, 2003)

| Origin | Name | Mw in kDa | Reference |
|---|---|---|---|
| *Metapenaeus ensis* (shrimp) | Met e 1; tropomyosin | 32.8 | Leung *et al.*, 1994 |
| *Homarus americanus* (American lobster) | Hom a 1; tropomyosin | 32.8 | Mykles *et al.*, 1998 |
| *Panulirus stimpsoni* (spiny lobster) | Pan s 1; tropomyosin | 32.8 | Leung *et al.*, 1998a |
| *Charybdis feriatus* (crab) | Cha f 1; tropomyosin | 32.8 | Leung *et al.*, 1998b |
| *Penaeus aztecus* (shrimp) | Pen a 1; tropomyosin | 36 | Daul *et al.*, 1994 |
| *Penaeus indicus* (shrimp) | Pen I 1; tropomyosin | 34 | Shanti *et al.*, 1993 |
| *Todarodes pacificus* (squid) | Tod p 1; tropomyosin | 38 | Miyazawa *et al.*, 1996 |
| *Haliotis midae* (abalone) | Hal m 1 | 49 | Lopata *et al.*, 1997 |

## 2.5 Cross-reactivity of Allergens

The structure of a protein is largely responsible for its cross-reactivity. Since the surface structure of a protein, particularly the epitope, is the most relevant site for antibody binding, both the primary (amino acid sequence) and tertiary (folding of protein) structures are the major determinants of protein cross-reactivity. Amino acid substitutions can markedly alter the outer protein surface, reducing the antibody reactivity of such a protein. In most situations cross-reactivity requires more than 70% amino acid identity, and decreases as the amino acid identity of two proteins become less. Therefore, cross-reactive proteins usually share a high degree of homology in terms of their primary and tertiary structures (Aalberse, 2000).

In 1966, it was reported that 62% of cod-allergic patients (n=100) reacted to all common fish species, including ones from foreign countries, which they had never been exposed to before, and 38% reacted to some while tolerating others (Aas, 1966). Since then, numerous studies have been conducted all over the world, investigating the IgE cross-reactivity of the same allergen in different species of fish, concentrating mostly on parvalbumin, which is the major allergen in fish.

In 1998, a group of scientists characterised the cross-reactive IgE-binding components in six different species of fish (cod, tuna, salmon, perch, carp and eel) by investigating the effects of reducing conditions on extracts, treatment of extracts with periodate, and depletion of $Ca^{2+}$ on the binding of IgE to the allergens. They subsequently reported that the sera from all patients allergic to fish (n=30), showed IgE reactivity to a 12 kDa protein, parvalbumin, in all 6 different extracts. Comparing the IgE

reactivity of extracts prepared under reducing conditions with ones prepared under non-reducing conditions, showed no difference, but the periodate treatment and $Ca^{2+}$ depletion led to a significant reduction of IgE binding. This concluded that parvalbumin was present in all extracts from the different species of fish, representing a cross-reactive fish allergen, with epitopes that are sensitive to periodate treatment and $Ca^{2+}$ depletion (Bugajska-Schretter *et al.*, 1998).

More recently, the allergenic cross-reactivity between nine commonly edible fishes was determined. The fish used in the study were cod, salmon, pollack, mackerel, tuna, herring, wolfish, halibut, and flounder. Sera were obtained from ten patients with a known allergy to fish, and were used together with rabbit anti-sera against Gad c 1 (cod), Sal s 1 (salmon), and The c 1 (pollack) in SDS-PAGE, IgE immunoblotting, IgG ELISA, and IgE ELISA inhibition experiments. Cod, salmon, pollack, and wolfish extracts showed an intensely stained 12 kDa protein, corresponding to parvalbumin on SDS-PAGE, whereas the parvalbumin band in herring was observed at 14 kDa. Halibut and mackerel showed faint bands at 12 kDa, whereas the parvalbumin band in tuna was extremely faint. On the immunoblots, halibut, mackerel and tuna showed very weak or almost invisible parvalbumin bands, whereas the rest demonstrated strong parvalbumin binding. On the IgE ELISA inhibition experiments, the percentage of recognition revealed that Gad c 1 was recognised by 100% of the sera, followed by The c 1, mackerel, and herring (90% of sera), whereas Sal s 1 was the least recognised. It was evident from the combined results of these experiments that Gad c 1, Sal s 1, The c 1, herring and wolfish contained the most potent cross-reacting allergens, while halibut, tuna, mackerel, and flounder showed much less allergenic reactions (Van Do *et al.*, 2005a).

## 2.6 Symptoms

Chemical mediators, which are released by tissue mast cells and blood basophils after the allergens cross-link IgE on these cells' surfaces, cause the clinical symptoms that are characteristic of allergy and allergic disease. The preformed mediators that are released from mast cell granules include histamine, neutral protease, heparin, eosinophil and neutrophil chemotactic factors, as well as platelet activating factors. Leukotrines, defined as metabolic products of arachidonic acid, which promote inflammatory processes, as well as prostaglandin PGD2 and thromboxanes are all newly synthesised prior to secretion. The normal function of these mediators is to help in the coordination of developing a defensive acute inflammatory reaction, but when these mediators are released in excess, such as in atopic disease, their effects become threatening (Roitt, 1997).

Due to the fact that the exposure to fish allergens can be through inhalation of airborne allergens during outdoor drying, skin contact while filleting and cooking fish, or ingestion of fish and products thereof, the symptoms of IgE-dependent food-allergic reactions can be classified according to the target organs that are affected (Aas, 1987). The typical organs affected are the skin, respiratory tract, gastrointestinal tract, and cardiovascular system and are discussed in more detail below.

### 2.6.1 The Skin

Handling food can elicit urticaria or atopic dermatitis (eczema) in hypersensitive individuals. Generalised urticaria, angioedema, and asthma can occur as a result of

cutaneous exposure to seafood (Bowman and Walzer, 1931). Although also occurring in consumers, skin reactions are often associated with occupational exposure (O'Neal and Lehrer, 1991).

### 2.6.2 The Respiratory Tract

Inhalation of vapours or steam generated by cooking any food containing allergens often causes asthma, rhinitis, laryngeal oedema, and rhino conjunctivitis. In some cases, respiratory symptoms occur following ingestion of allergenic foods (O'Neal and Lehrer, 1991).

### 2.6.3 The Gastrointestinal Tract

Ingestion of allergens by hypersensitive individuals has shown localised or generalised itching of the buccal mucosa (pruritus), as well as erythematous cutaneous lesions (urticaria) and swelling of the face or tongue (angioedema). More commonly occurring symptoms are nausea, vomiting, and diarrhoea (O'Neal and Lehrer, 1991).

### 2.6.4 The Cardiovascular System

Highly sensitive individuals may experience anaphylactic shock when exposed to the relevant allergens, which is the most severe and clinically urgent allergic reaction. This shock is usually of a multi-system nature, often including symptoms described for the skin, respiratory system, and the gastrointestinal system, in addition to possible

hypotension, electrocardiogram (EKG) changes, and ultimately cardiovascular collapse (Brunner and Waltzer, 1928).

## 2.7 Diagnosis of Food Allergy

The diagnosis of food allergy starts with conducting a thorough medical history and physical examination. It is important to determine if the suspected adverse reaction is to food and whether it is IgE-mediated or not. This is mainly achieved with the patient's recollections of events leading to the development of symptoms, which are observed on the physical examination. On establishing the nature of the symptoms to be of either IgE- or non IgE-mediation, the most appropriate test will be performed to determine the causative allergen, and to treat the patient accordingly, as explained by the flow-chart in Figure 2.10 (Sampson, 1999).

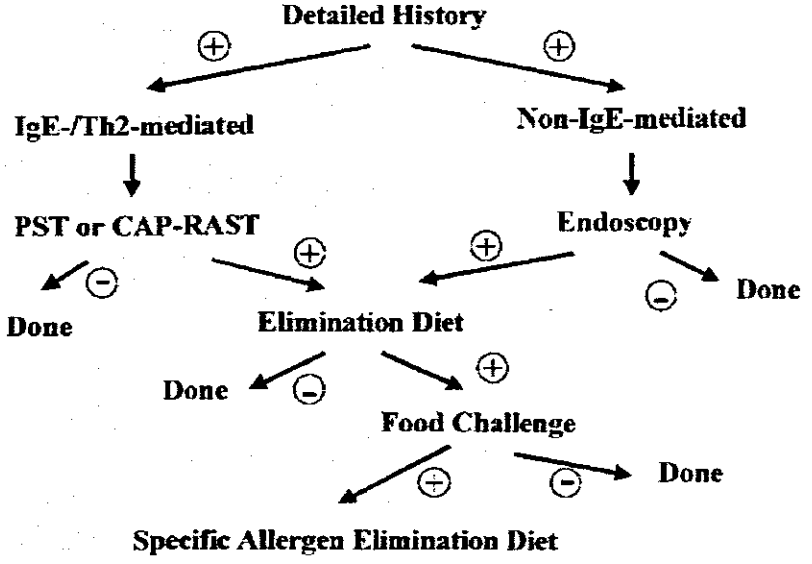


**Figure 2.10. Diagnostic approaches for food allergy evaluation.** PST (Prick Skin Test) is the same as Skin prick test (SPT). When IgE-mediated food allergy is expected, SPT or RAST is performed, followed by elimination diets and food challenges, resulting in specific allergen elimination from diets. Non IgE-mediated food allergies are usually evaluated by endoscopy, followed by elimination diets and food challenges in order to determine the allergen to be eliminated from the diet (Sampson, 1999).

### 2.7.1 Double Blind Placebo-controlled Food Challenges

Double blind placebo-controlled food challenges (DBPCFCs) are oral challenges performed on the patient, with neither patient nor physician knowing the contents of the challenge. This test is considered the "gold standard" for the diagnosis of food allergies, and is usually used for research studies, for chronic disorders, multiple allergies, and in situations where patients' biased perceptions need to be eliminated (Sampson, 1999). For the evaluation of delayed allergic reactions, oral challenges can be conducted safely on an outpatient basis, otherwise it can be performed in a clinic or hospital in the presence of trained personnel and equipment for treating life-threatening conditions, such as a systemic anaphylaxis that may arise from an oral challenge (Joint Task Force on Practice Parameters, 1998).

### 2.7.2 IgE-mediated Disorders

#### (i) Skin Prick Testing

Skin prick tests (SPT) are performed by applying a glycerinated food extract, as well as appropriate positive (histamine) and negative (saline) controls, on the skin of a patient and piercing into the skin. Wheals of at least 3 mm larger than those induced by the negative control, within 15 minutes of extract application, are considered positive. All skin pricks less than that is considered negative (Sampson, 1999). A Negative SPT confirms the absence of an IgE-mediated allergic reaction to the tested extract with a negative predictive accuracy of >95%, whereas a positive SPT does not necessarily prove that the extract is causal of an allergic reaction. Moreover,

the wheal size has been roughly correlated with the likelihood of clinical allergic symptoms (Sporik, 2000). Preferably, fresh foods must be used to perform SPT rather than commercial extracts. Previously, Herian *et al* confirmed the absence of several major soybean proteins when commercial extracts were used for SPT instead of fresh foods. The immunoblots with soy-allergic sera also indicated alterations, reductions, or losses of IgE-binding in commercial allergen extracts, compared to extracts of soy flour (Herian *et al.*, 1992).

### (ii) Radio Allergosorbent Test

In radio allergosorbent tests (RASTs), the allergen of interest is bound to a solid matrix and exposed to the patient's serum. An IgE antibody specific to the allergen binds to the protein-matrix and is detected by a labelled secondary antibody specific to the IgE. RASTs demonstrate a similar sensitivity and specificity pattern as SPTs when a RAST score of 3 or greater is considered (Sampson and Albergo, 1984), however it is preferable to use RAST in clinical situations where patients present with significant dermatographism, severe skin disease with limited surface area for testing, difficulty in discontinuing antihistamines, and suspected exquisite sensitivity to certain foods (Sampson, 1999).

### (iii) Basophil Histamine Release Assay

The measurement of basophil histamine release (BHR) was not widely employed in routine diagnostic laboratories, because it was time consuming, labour intensive and expensive to maintain. The lack of standardisation of the methodology, as well as the

issues regarding quality assurance also contributed to BHR assays not being routinely used, but with the introduction of more commercial BHR assays, it is now being utilised more commonly in the routine setting (Crockard and Ennis, 2001).

### 2.7.3 Non IgE-mediated Disorders

A number of non-specific laboratory tests are abnormal in non IgE-mediated food-induced allergic disorders, but none of these tests have been proven to identify the foods responsible for these reactions. Peripheral blood eosinophilia is suggestive of allergic eosinophilic gastroenteritis (Min and Metcalfe, 1991), while an increase in the neutrophil count with a 'left-shift' is often seen in patients with dietary protein-induced enterocolitis who recently experienced an allergic reaction (Powell, 1986). Similarly, other non IgE-related tests which are often promoted to be useful for diagnosing food allergy are not supported by scientific testing, and should preferably be avoided. These tests include food-specific IgG or IgG4 antibodies, provocation-neutralisation testing and applied kinesiology (Terr *et al.*, 1996).

## 2.8 Treatment of Food Allergy

The only proven therapy for food hypersensitivity is the strict elimination of the offending allergen (Sicherer and Sampson, 2006). Otherwise, medication is available for the treatment of symptoms already caused by food allergens. Antihistamines are generally used to reduce itching and rash, while epinephrine is considered the primary treatment for anaphylaxis. Other methods of allergy-symptom treatments include cardiopulmonary resuscitation, corticosteroids, oxygen, intravenous fluids, inhaled

bronchodilators, and medications to support blood pressure (Joint Task Force on Practice Parameters, 1998).

More recently, a review by Nieuwenhuizen and Lopata (2005) on fighting food allergy indicated that specific as well as non-specific immunotherapies are being developed to treat food allergies. Apart from using recombinant proteins and peptides, specific immunotherapy utilise immunostimulatory DNA sequences to reduce the sensitivity to allergens by switching from a Th2 type response to a Th1 or regulatory type response. Generally, specific immunotherapy produces a considerable amount of side-effects, which are currently being reduced with a better understanding of the pathophysiology and immunologic mechanisms of food allergies, together with the molecular characterisation of food allergens.

Non-specific immunotherapy consists of anti-IgE administration, cytokine therapy and natural therapies, such as probiotics and Chinese herbal medications. These approaches of treatment are useful to help abrogate the symptoms and possibly minimising the side-effects related with conventional immunotherapy (Nieuwenhuizen and Lopata, 2005).

# Chapter 3

# Materials and Methods

## 3.1 Collection of Samples

Fresh and frozen pilchard (*Sardinops sagax*) specimens were collected from a factory in Hout Bay, Cape Town. The samples were transported on ice to the laboratory and production of protein extracts performed on arrival at the laboratory.

Similarly, tuna (*Thunnus albacares)*, hake (*Merluccius* spp), snoek (*Thyrsites atun),* yellow tail (*Seriola lalandi)*, Alaska pollack *(Theragra chalcogramma)*, and anchovy (*Engraulis capensis)* were collected and transported to the laboratory on ice.

Canned pilchard was collected directly from a seafood processing factory in St. Helena Bay, Western Cape, South Africa. Prior arrangements were made with the cannery to provide the sealed canned pilchard after the cooking stage, but before any sauce was added. They were transported to the laboratory on ice and processed immediately upon arrival at the laboratory.

Additionally, blood was collected from 10 seafood processing workers at two pilchard processing plants in the Western Cape. These workers demonstrated clinical symptoms of occupational allergy, as summarised in Table 3.1. They signed the consent forms with the guidance of a nurse, who collected the blood and sent it to the

laboratory where it was centrifuged to separate the serum. IgE-mediated fish allergy was verified by subsequent determination of pilchard and anchovy-specific IgE CAP RAST analysis (Pharmacia, Uppsala, Sweden), previously performed in the laboratory, (see Table 3.1), before aliquots of the serum were stored frozen at -80°C until use (Jeebhay, 2003).

**Table 3.1 Serological and clinical data of the fish processing workers**
(Jeebhay, 2003)

| Worker ID | IgE-Specific CAP RAST (kUA/L) | | SPT | | Clinical Symptoms | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pilchard | Anchovy | Pilchard | Anchovy | Wheeze | Hay fever | Eye-wrk | Skin-wrk | Chest-wrk |
| A | 17.3 | 25.1 | Not Done | Not Done | + | + | + | + | + |
| B | <0.35 | 0.42 | Not Done | Not Done | + | + | - | - | + |
| C | 0.39 | 0.38 | + | - | - | - | - | - | - |
| D | 0.49 | 0.81 | - | - | - | - | - | - | - |
| E | 0.48 | 0.66 | - | - | + | + | + | + | + |
| F | 0.47 | 0.99 | Not Done | Not Done | - | - | - | + | - |
| G | 1.12 | 1.23 | Not Done | Not Done | + | - | - | - | + |
| H | 0.52 | 0.73 | - | - | - | - | - | - | - |
| I | 0.48 | 0.93 | + | + | - | - | + | - | - |
| J | 9.47 | 2.13 | + | + | + | + | + | + | + |

SPT = Skin Prick Test (Not performed on Workers A, B, F and G)
wrk = work-related
kUA/L = kilo units antigen per litre

The rabbit antisera were produced by immunising two New Zealand rabbits, each with an extract from either canned pilchard (*Sardinops sagax*) or fresh Cape anchovy

(*Engaulis capensis*). After the initial subcutaneous injections, immunisations were performed with the same extract concentrations in incomplete Freund's adjuvant at 3 and 6 weeks, respectively, after the first immunisations. The blood samples were collected at 8 weeks, centrifuged, and sera stored at -20°C until use (Lopata *et al.*, 2005).

## 3.2 Protein Extract Production

Upon arrival at the laboratory, the head, internal organs, and caudal fin of the fresh pilchard and anchovy were removed first, using sterile disposable scalpels (RIBBEL, Germany). All other types of fish were collected without heads. Following that, the skin and bones were removed from the fillet before chopping the latter into fine pieces. Phosphate buffered saline (PBS) (Appendix1.1) was added to the finely chopped fish in a 1:3 w/v ratio, and homogenised (Ultra-Turrax T25, Germany), before agitation at 4°C overnight. The mixture was further homogenised and centrifuged (Beckman J2-21M, USA) at 1000g for 30 minutes, after which the supernatant was removed and frozen at -20°C overnight. Following thawing of the supernatant, it was centrifuged (Beckman TJ-6, UK) at 200g for 5 minutes and the intermediate layer between the fatty top layer and the pellet was removed. This layer was centrifuged (Beckman J2-21M, USA) at 1000g for 30 minutes, and the supernatant filtered as follows: Pre-filtration using Whatman paper of 11 µm pore size, followed by a 8.0 µm filter (Sartorius, Germany), and a sterile filter of 0.45 µm (Millipore, USA) by vacuum (Neuberger, N022AN.18, Germany). The purified extract was stored in aliquots at -80°C until further use.

## 3.3 Total Protein Estimation

Total protein estimation was done on each protein extract as specified in the Bicinchoninic acid assay (BCA) kit (Pierce, USA). Briefly, protein standards and extracts were diluted with PBS, and 10 µl of each sample was pipetted in duplicate into wells on a MaxiSorp (Nunc, Denmark) microplate. Hundred microlitres of PBS was used as a sample blank. A BCA working solution was prepared by mixing solution A and solution B at a ratio of 50:1, and 200 µl of this solution was added to each standard dilution, extract sample and sample blank wells. The plate was incubated at 37°C for 30 minutes, cooled to room temperature, and absorbance determined at 540 nm (Versa Max Micro plate reader, Labotech, USA).

## 3.4 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

### 3.4.1 Preparation of 5-16% Gradient Gels

Glass plates were cleaned with 70% ethanol and distilled water ($dH_2O$), and assembled using sealing agar (Appendix 1.2) to seal the bottom and sides of the plates. Five percent and 16% polyacrylamide mixtures were prepared as set out in Table 3.2. After preparation of these mixtures, the solutions were vortexed (Vortex-Genie 2, Scientific industries, USA) and the 16% gel was first poured into the mixing chamber, followed by the 5% gel that was poured in the reservoir chamber of the gradient gel apparatus. Thirty microlitres of 10% Ammonium Persulfate (AMPS) (Appendix 1.4) was added directly to the mixing chamber, after which the stirrer was switched on, the interconnecting valve opened, and suction applied to the tubing at the

outlet. The vertical gel unit was filled with this gradient solution, ranging from 5-16%.

Isopropanol was used to overlay the gel for at least 1 hour, which was then removed and the gel surface rinsed with water. A 1:4 diluted 2.5x separating buffer (Appendix 1.3) was used to overlay the gel overnight at room temperature, covered with aluminium foil to avoid condensation. After the gel polymerised, the separating buffer was rinsed off with distilled water and a stacking gel (Table 3.3) was poured onto the separating gel. A comb was inserted into the stacking gel, and the gel was allowed to polymerise for about 20 minutes. It was then placed into an electrophoresis tank and filled with SDS-PAGE electrophoresis buffer (Appendix 1.5). The comb was removed and the wells rinsed out with electrophoresis buffer, using a sterile syringe and needle (Omnican, Braun, Switzerland).

**Table 3.2 Reagents for gradient gel preparation**

| **Reagents** | **5% Polyacrylamide gel** | **16% Polyacrylamide gel** |
|---|---|---|
| **30% Acrylamide**<br>30% w/v Acrylamide Bis solution (Sigma-Aldrich, USA) | 0.83 ml | 2.64 ml |
| **2.5x Separating buffer**<br>(Appendix 1.3) | 1.99 ml | 1.99 ml |
| **Distilled Water** | 2.14 ml | 0.34 ml |
| **TEMED**<br>N, N, N', N', - Tetramethyl-Ethylenediamine (Promega, USA) | 4.5 µl | 4.5 µl |
| **10% AMPS**<br>Ammonium Persulfate (Biorad, USA) | 45 µl | 30 µl (directly into mixing chamber) |

### 3.4.2 Preparation of Samples and SDS-PAGE

For SDS gels, which only needed to be stained with Coomassie Brilliant Blue dye (Appendix 1.6), 15 µg of each protein extract was combined with 4 µl of 5x sample buffer (Appendix 1.7) and made up to a total volume of 20 µl with PBS. This mixture was vortexed, heated to 95°C for 5 minutes in a heating block (Dry Block Heater HB2, Hagar Designs, South Africa), and centrifuged at 3000g for 10 seconds before it was loaded into the wells of the stacking gel. Precision Plus Protein Standard (Biorad, USA) was used as molecular weight marker.

Electrophoresis was performed at 90 V until the dye front reached the bottom of the separating gel (about 3 hours), after which the gel apparatus was disassembled. The gel was stained with Coomassie Brilliant Blue dye while agitating on a platform shaker (Stuart Scientific, UK) for 1 hour, before it was left gently agitating in a destaining solution (Appendix 1.8) overnight.

## 3.5 Immunoblotting

For immunoblotting, 5 µg of the protein extract, mixed with 4 µl 5x sample buffer (Appendix 1.7) and made up to 20 µl with PBS, was loaded in a gel. SDS-PAGE was performed as described above, but instead of staining the gel, it was prepared for Western blotting by soaking it in transfer buffer (Appendix 1.10) for 5 minutes. A Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham, UK), was pre-treated in methanol for 5 minutes, rinsed with distilled water, and kept at 4°C in transfer buffer for at least 15 minutes prior to the transfer process. Two fibre pads and

8 pieces of 3 mm filter paper (Whatman, England) were soaked in transfer buffer at 4°C prior to assembling for immunoblotting.

**Table 3.3 Stacking gel preparation**

| Reagent | Volumes |
|---|---|
| **30% Acrylamide**<br>30% w/v Acrylamide Bis solution (Sigma-Aldrich, USA) | 0.56 ml |
| **5x stacking buffer**<br>(Appendix 1.9). | 0.66 ml |
| **Distilled Water** | 2.0 ml |
| **TEMED**<br>N, N, N', N', - Tetramethyl-Ethylenediamine (Promega, USA) | 4.0 µl |
| **10% AMPS**<br>Ammonium Persulfate (Biorad, USA) | 30 µl |

A Mini Trans-Blot Electrophoretic Transfer Cell (Biorad, USA) was assembled as depicted in Figure 3.1, by placing the gel holder cassette with the black side down on a clean, flat surface. One pre-wetted fibre pad, followed by 4 layers of pre-wetted filter paper was placed on the cassette, rolling out any air bubbles using a disposable pipette. The gel was placed on top of the filter paper, followed by the membrane. Finally, 4 filter papers and 1 fibre pad were placed on top and the cassette was closed.

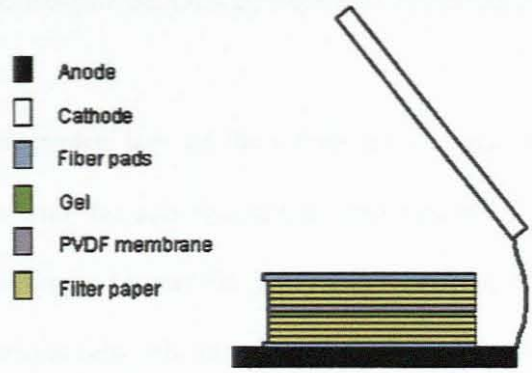


**Figure 3.1. The Mini Trans-Blot Electrophoretic Transfer Cassette was assembled in a sandwich format as illustrated here.**

Aligning the black side of the cassette and the black side of the negative electrode, the cassette was placed in the power module and immersed in the buffer tank. A frozen Bio-Ice cooling unit was placed inside the buffer tank and the chamber was added with transfer buffer. The power supply was connected and protein transfer proceeded at 400mA for 1 hour, after which the blotting sandwich was disassembled. The gel was stained in Coomassie Blue for 1 hour and destained overnight, to assess if all proteins were transferred from the gel to the membrane. The membrane was then removed for further processing.

### 3.5.1 Assessment of Protein Transfer Efficiency

Before performing the immunoblotting experiments with the relevant antibodies, one membrane was assessed for the efficient transfer of proteins. This was achieved by placing the PVDF membrane following the transfer in Coomassie stain for 20 minutes, after which it was destained overnight at room temperature, and stored in tissue paper to dry. This step was performed once only after the transfer process was optimised.

### 3.5.2 Monoclonal Anti-Parvalbumin Immunoblotting

The molecular weight marker lane on the membrane was cut off and stored in tissue paper at room temperature for subsequent use. The remainder of the membrane was placed in blocking solution (Appendix 1.11) for 1 hour at room temperature. The primary antibody, anti-parvalbumin monoclonal antibody (Sigma, Israel) was diluted 1 in 8000 with 1% fat-free milk powder (Farmers' Pride, South Africa), made up in 10 ml Tris Buffered Saline (TBS) (Appendix 1.12), added to the membrane, and placed on a platform shaker for 30 minutes at room temperature. Following that, the membrane was washed, first for 10 minutes in 50 ml TBS, while gently shaking, then twice in 50 ml TBS for 5 minutes at a time.

The secondary antibody, goat anti-mouse IgG1-Alkaline Phosphatase-labelled (AP) (Southern Biotechnologies, USA), diluted 1 in 2000 with 1% milk powder in 10 ml TBS with 0.5 % Tween 20 (Saarchem, SA), was added to the membrane and incubated on a platform shaker for 30 minutes at room temperature. The membrane was washed with washing buffer (Appendix 1.12) for 10 minutes, followed by 2 washes of 5 minutes each in fresh washing buffer. The substrate solution was made up by dissolving 1 Nitro Blue Tetrazolium (NBT) tablet (Sigma, Germany) in 10 ml distilled water ($dH_2O$). It was then added to the membrane and agitated for approximately 7 minutes, until the purple-coloured antigenic bands became visible, and removed before background developed. The membrane was rinsed in distilled water and left in tissue paper to dry and stored at room temperature.

### 3.5.3 Rabbit Anti-Pilchard Immunoblotting

The membrane containing the transferred protein bands was blocked for 1 hour as described in section 3.5.1. Rabbit anti-pilchard serum, the primary antibody, was diluted 1 in 8000 with TBS containing 1% milk powder. This diluted serum was added to the membrane and incubated at room temperature for 30 minutes on a platform shaker. Following this, the membrane was washed as described above (Section 3.5.2), and goat anti-rabbit IgG heavy and light chain-AP (Southern Biotechnologies, USA) was diluted 1 in 2000 with 1% milk powder in 10 ml TBS with 0.5% Tween 20, and incubated with the membrane. Membrane washing was repeated as stated above (Section 3.5.2). A NBT tablet dissolved in 10 ml $dH_2O$ was used as substrate, and incubated with the membrane until purple-coloured antigenic bands were detected on the membrane. Before the background developed, the reaction was stopped by rinsing the membrane with $dH_2O$ and it was left in tissue paper to dry.

### 3.5.4 Rabbit Anti-Anchovy Immunoblotting

The membrane was blocked for 1 hour as described before, followed by incubation with rabbit anti-anchovy serum, diluted 1:8000 in TBS containing 1% milk powder at room temperature for 30 minutes on a platform shaker. The washing was done as explained above (Section 3.5.2), and goat anti-rabbit IgG heavy and light chain-AP (Southern Biotechnologies, USA) was diluted 1 in 2000 with 5% milk powder in 10 ml TBS containing 0.5% Tween 20, and incubated with the membrane. Washing was repeated as stated in Section 3.5.2. A NBT tablet dissolved in 10 ml $dH_2O$ was used as the substrate and added to the membrane until purple antigenic bands were

visible on the membrane, after which the reaction was stopped before background developed.

## 3.6 Immunoblotting with Sera from Workers

After preparing and pouring the gradient gel as described in Section 3.4, a slot immunoblot comb as shown in Figure 3.2 (on the right hand side) was inserted into the stacking gel after which the gel was allowed to polymerise. One small and another large well were consequently formed by this comb at the stacking gel surface for the loading of samples onto the gel.

Samples were prepared by diluting 50 µg of protein extracts of fresh, frozen and canned pilchard with 40 µl of 5x sample buffer and made up to 200 µl with PBS. The extract dilutions were vortexed, heated to 95°C for 5 minutes, centrifuged at 3000g for 10 seconds, and loaded onto the stacking gel. For this purpose, 200 µl of each extract dilution was loaded into the large well, and 5 µl of the molecular weight marker was loaded into the smaller well. SDS-PAGE and transfer proceeded as described before (Sections 3.4 and 3.5), after which the membrane was placed in 5% blocking solution for 45 minutes.

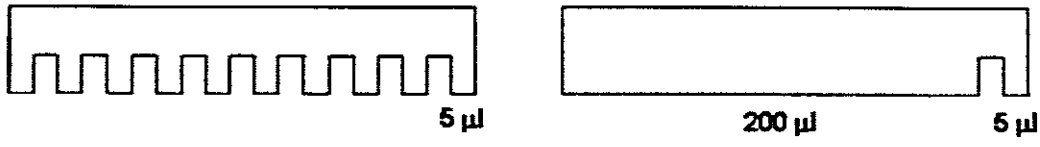


**Figure 3.2. Gel combs used to form the wells for sample loading onto gels.** The comb on the left, which only makes provision for the loading of 5 µl in each of ten wells, is used for ordinary immunoblots. Slot immunoblotting experiments were performed with the comb on the right, made of a 200 µl and a 5 µl well. The extract dilutions were loaded into the larger well, whereas the molecular weight marker was loaded in the 5 µl well.

### 3.6.1 IgG Slot Immunoblots

A slot immunoblot apparatus, having multiple parallel slots, was fixed to the blocked membrane, using clamps on all sides to make certain that no intra-slot leakage would occur. One millilitre of workers' sera, diluted 1:1000 with 1% milk powder in TBS, were added into each slot of the immunoblot apparatus onto the membrane and incubated for 1 hour at room temperature. Thereafter, each lane of the membrane was washed with 1x TBS while the apparatus was still assembled. Following primary wash, the apparatus was disassembled and the blot was washed a further 3 times for 10 minutes at a time in washing buffer (Appendix 1.13). Thereafter, the membrane was incubated with 1:1000 diluted goat anti-human IgG (Kirkegaard + Perry Laboratories, USA) for 1 hour at room temperature before it was washed 3 times for 10 minutes as described before (Section 3.5.2). NBT substrate was added and the purple colour development of the antigenic bands observed after about 7 minutes before the background colour developed. The membrane was rinsed in $dH_2O$ and stored in tissue paper to dry.

### 3.6.2 IgE Slot Immunoblots

Similarly, as described in Section 3.6.1, a slot blot apparatus was attached to a blocked membrane. Each slot of the apparatus was loaded with 1 ml of the 10 workers' sera, diluted 1:20 with 1% milk powder in TBS and allowed to be in contact with the membrane overnight at 4°C, followed by washes as described before in Section 3.6.1. (Serum from worker B was insufficient; therefore no serum was loaded on immunoblots for fresh and frozen pilchard IgE). Goat anti-human IgE

(Kirkegaard + Perry Laboratories, USA) was diluted 1:1000 and the membrane was incubated with it for 1 hour at room temperature, while gently agitating. Washes were performed as described before (Section 3.5.2), and NBT substrate added to the membrane for colour development. The membrane had to be closely observed for approximately 1 hour so that colour development could be terminated prior to excessive background development. This was done by rinsing the membrane with $dH_2O$, after which it was dried and stored in tissue paper at room temperature.

# Chapter 4

# Results

## 4.1 Total Protein Concentrations

Protein was extracted from fillets of fresh, frozen and canned pilchard, as well as anchovy, tuna, yellow tail, hake, snoek, and pollack. Before purified specimens were aliquoted, the total protein concentration for each was determined spectrophotometrically using a BCA kit as specified by the manufacturer (Pierce, USA). The average of all readings that fell within the standard curve was taken as the protein concentrations of the extracts and is summarised in Table 4.1. The total protein concentrations ranged from 2.35 mg/ml in hake to 9.54 mg/ml in frozen pilchard.

## 4.2 SDS-PAGE Gel of Extracts

Following denaturing gel electrophoresis, the molecular weight marker of the gel in lane 1 demonstrated protein sizes ranging from 10 to 250 kDa. All the fish extracts showed a similar-sized protein band of approximately 12 kDa, as indicated by the arrow in Figure 4.1. The 12 kDa band is the approximate molecular weight of parvalbumin, a possible allergen in pilchard. Tuna and hake showed faint bands around 12 kDa, whereas all the other extracts demonstrated strong, prominent bands.

**Table 4.1 Total protein concentration of fish samples**

| Fish Protein Extract | Grams of Fillet Used | Total Protein Concentration |
|---|---|---|
| Fresh Pilchard | 250 g | 4.23 mg/ml |
| Frozen Pilchard | 77 g | 9.54 mg/ml |
| Canned Pilchard | 48 g | 3.10 mg/ml |
| Anchovy | 103 g | 7.45 mg/ml |
| Tuna | 85 g | 5.60 mg/ml |
| Yellow Tail | 143 g | 7.40 mg/ml |
| Hake | 139 g | 2.35 mg/ml |
| Snoek | 124 g | 8.23 mg/ml |
| Pollack | 207 g | 4.70 mg/ml |

Except for canned pilchard, all extracts had prominent protein bands between 24 and 48 kDa. Canned pilchard had a very strong band at about 12 kDa, and fainter bands at 36, 120, 250 kDa and above. All of the fish extracts also showed the presence of a 36 kDa protein, although faint in canned pilchard, yellow tail and snoek. In 7 of the extracts, a protein band was also present close to 84 kDa. Apart from canned pilchard, snoek was the only other extract demonstrating a protein band at 120 kDa.

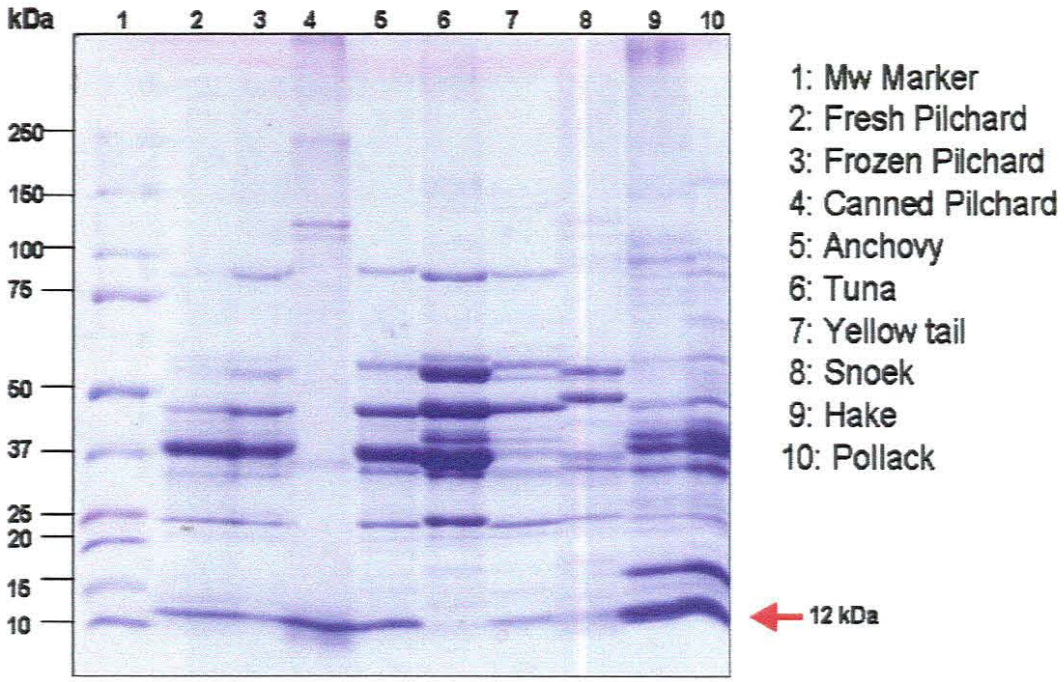


**Figure 4.1. SDS-PAGE of fish extracts.** Nine fish extracts were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1 was loaded with a molecular weight marker and lanes 2-10 as listed to the right of the gel. The arrow indicates the position of parvalbumin.

## 4.3 Assessment of Protein Transfer

A polyacrylamide gel was loaded with 50 µg fresh pilchard extract and denaturing protein electrophoresis was performed. Following transfer of the proteins from the gel to a PVDF membrane, the membrane was stained with Coomassie Brilliant Blue to ascertain transfer efficiency.

The molecular weight marker bands ranged from 10-150 kDa, and the proteins from the fresh pilchard were separated between 10 and 84 kDa as shown in Figure 4.2. As expected, it was concurred that the process of transferring proteins from the gel to the PVDF membrane was successful, especially over the 10 – 84 kDa size range.

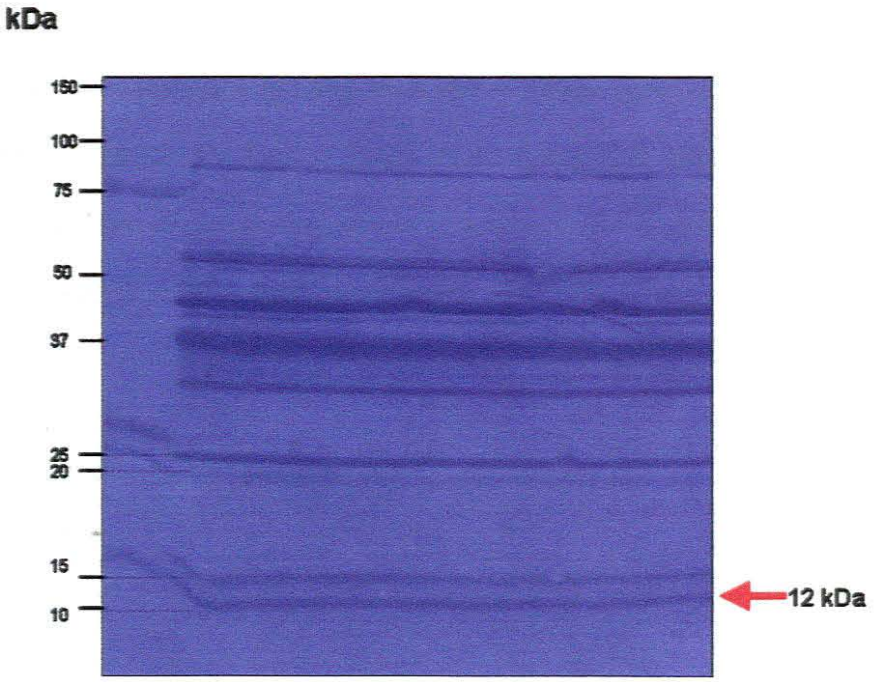


**Figure 4.2. Protein transfer assessment.** A molecular weight marker was loaded in the left-hand lane and fresh pilchard on the rest of the gel. In this stained membrane it is evident that the transfer of proteins of the fresh pilchard extract from a gel was successful. Bands ranging between 10 and 85 kDa in molecular weight were transferred efficiently. The arrow on the right-hand side of the membrane indicated the 12 kDa protein band, which is the molecular weight size of parvalbumin.

## 4.4 Anti-Parvalbumin Reactivity to Fish Extracts

To demonstrate binding of the fish extracts with a monoclonal anti-parvalbumin antibody, a molecular weight marker was loaded in lane 1, and different fish extracts in lanes 2 through 10, as depicted in Figure 4.3. Generally, a protein in the region of 12 kDa in all fish extracts demonstrated binding to the anti-parvalbumin antibody. Tuna and snoek showed very faint bands around 12 kDa, whereas frozen pilchard had the most prominent band in that region, even though equivalent amounts of protein were separated on the initial gel. Frozen pilchard, anchovy, and pollack showed additional larger bands at about 24 kDa.

With the exception of fresh and canned pilchard, snoek, hake, and pollack, all other fish extracts demonstrated binding with this antibody around 36, 48 and 60 kDa, respectively. The 48 kDa band in frozen pilchard appeared more prominent than the rest of the extracts demonstrating a band of that size. The frozen pilchard extract also revealed an additional faint band in the region of 60 kDa.

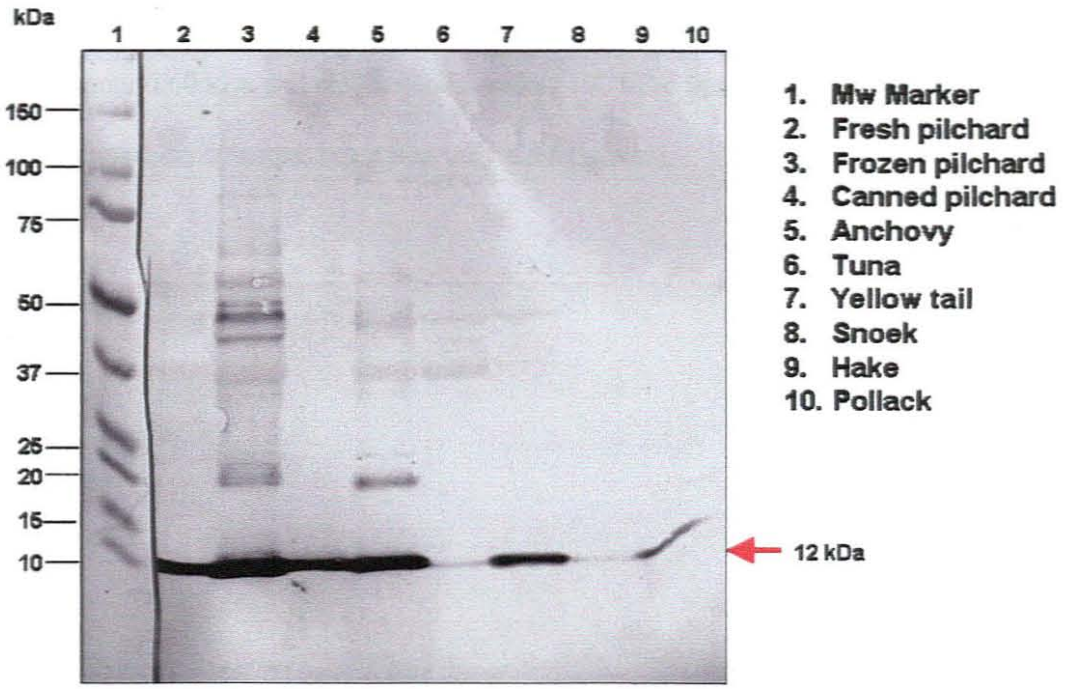


**Figure 4.3. Anti-parvalbumin reactivity of fish extracts.** A gel was loaded with 9 different fish extracts, separated by denaturing electrophoresis, transferred to a membrane and probed with anti-parvalbumin. The molecular weights are indicated on the left-hand side of the membrane and the rest of the lanes were loaded as shown on the right. The arrow on the right-hand side of the membrane indicates the location of a 12 kDa protein band, which is putatively parvalbumin.

## 4.5 Anti-Pilchard Reactivity to Fish Extracts

The reactivity of fish extracts to anti-pilchard antibody was demonstrated by exposing a membrane with separated proteins from all the extracts to this antibody. In Figure 4.4 it is shown that a 12 kDa protein was detected in all the fish extracts,

except in the tuna extract. The snoek extract showed a weak band in this region, with canned pilchard and anchovy demonstrating the strongest binding. All extracts, except canned pilchard, showed the presence of a 24 kDa protein. This protein was very weakly detected in hake and pollack extracts. Fresh and frozen pilchard, tuna, yellow tail and snoek extracts showed a faint band around 36 kDa. With the exception of canned pilchard, hake and pollack, a protein was detected in the region of 48 kDa, whereas tuna and yellow tail extracts showed the presence of reactive protein double bands around 60 kDa and single bands around 100 kDa. In the snoek extract, a 60 kDa protein was also detected, but it was only a single band.

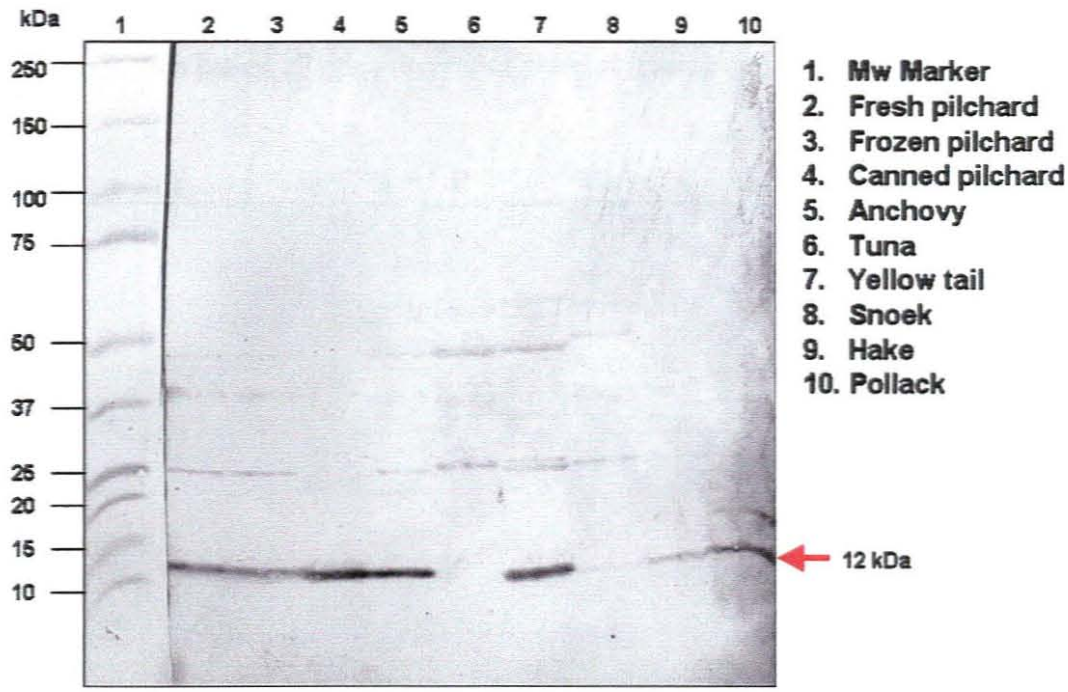


**Figure 4.4. Rabbit anti-pilchard reactivity of fish extracts.** Fish protein extracts were separated and immunoblotted with a polyclonal rabbit anti-pilchard primary and goat anti-rabbit secondary antibody. NBT/BCIP substrate was used to detect the AP-label on the bound antibodies. The molecular weight is indicated on the left with the molecular weight marker in lane 1 and the extracts as specified in lanes 2-10. The red arrow on the right-hand side of the membrane indicates the 12 kDa band, which is the size of parvalbumin band.

## 4.6 Anti-Anchovy Reactivity to Fish Extracts

During the investigation of anti-anchovy reactive proteins in all the fish protein extracts, it was observed that a protein around 12 kDa was the most prominent anti-anchovy binding protein, as indicated by the arrow in Figure 4.5. Tuna and hake extracts showed the weakest binding in the 12 kDa region, while canned pilchard, anchovy and yellow tail showed the strongest binding affinity. Frozen pilchard, anchovy, tuna, yellow tail and snoek extracts additionally demonstrated very weak bands around 36 kDa, whereas the same extracts, except for frozen pilchard, showed protein binding in the region of 48 kDa and approximately 84 kDa. Except for the 12 kDa protein bands, all other detectable proteins reacted only weakly.

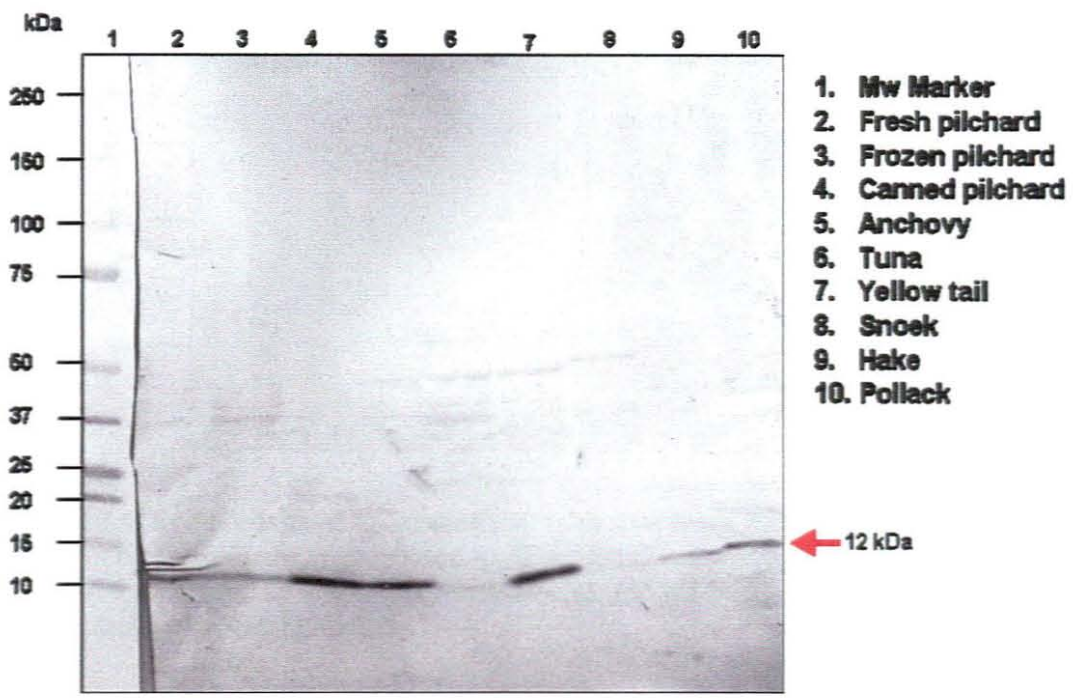


**Figure 4.5. Rabbit anti-anchovy reactivity of fish extracts.** Following SDS-PAGE, the separated proteins were transferred onto a PVDF membrane and exposed to a rabbit anti-anchovy antibody. The molecular weight marker is shown in lane 1 and lanes 2-10 represent the different fish protein extracts. The arrow on the right-hand side of the membrane indicates the putative parvalbumin bands, found around 12 kDa.

## 4.7 Fresh Pilchard Anti-Human IgG- and IgE-Reactivity

### 4.7.1 Reactivity with Anti-Human IgG

A fresh pilchard protein-containing membrane was exposed to sera of 10 fish-allergic workers, using a slot blot apparatus. The IgG antibodies of the workers' sera formed complexes with some membrane-bound pilchard proteins as seen in Figure 4.6. Prominent bands were observed between 24 and 48 kDa throughout all the sera, whereas fainter bands were observed between 24 and 12 kDa, and again between 48 and 120 kDa.

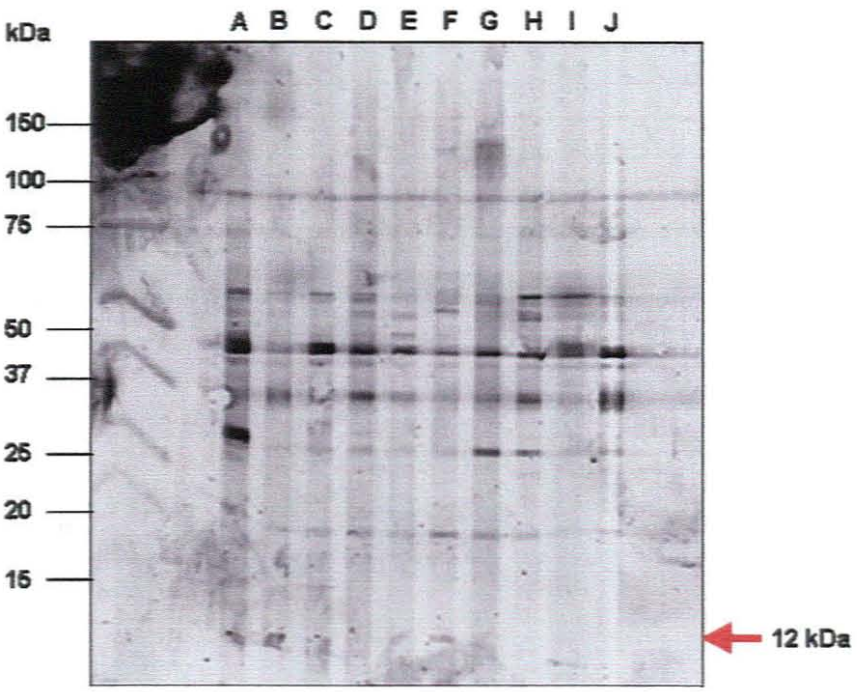


**Figure 4.6. Fresh pilchard IgG-reactivity with sera from fish-allergic workers.** This slot blot membrane was exposed to 10 workers' sera and a goat anti-human IgG. Lanes A-J represent the individual workers' sera, and the bands on the extreme left are the molecular weight markers.

As seen in Table 4.2, sera from workers A, B, C, E, F, I and J demonstrated binding to the fresh pilchard proteins of 12 kDa in molecular weight. All sera recognised a 24 kDa protein, as well as proteins around 36, 48, 60 and 84 kDa. Figure 4.7, which illustrates the frequencies of IgG-binding to pilchard proteins showed that from all the IgG-reactive proteins of the fresh pilchard extract, the 120 kDa protein was the least

reactive, being bound by 2 of the 10 sera, while the proteins at 24, 36, 48, 60 and 84 kDa were all reacting stronger with the IgG antibodies in the fish-allergic workers' sera. The 12 kDa protein was bound by 7 of the 10 sera.

**Table 4.2 Estimated IgG-reactive protein molecular weights in fresh pilchard**

| Molecular Weight in kDa | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| >250 | - | - | - | - | - | - | - | - | - | - |
| 250 | - | - | - | - | - | - | - | - | - | - |
| 150 | - | - | - | - | - | - | - | - | - | - |
| 120 | - | - | - | - | - | X | X | - | - | - |
| 84 | X | X | X | X | X | X | X | X | X | X |
| 72 | X | - | - | - | - | - | X | X | - | X |
| 60 | X | X | X | X | X | X | X | X | X | - |
| 48 | X | X | X | X | X | X | X | X | X | X |
| 36 | X | X | X | X | X | X | X | X | X | X |
| 24 | X | X | X | X | - | - | X | X | - | X |
| 20 | - | - | - | - | - | - | - | - | - | - |
| 12 | X | X | X | - | X | X | - | - | X | X |

X = present
- = absent

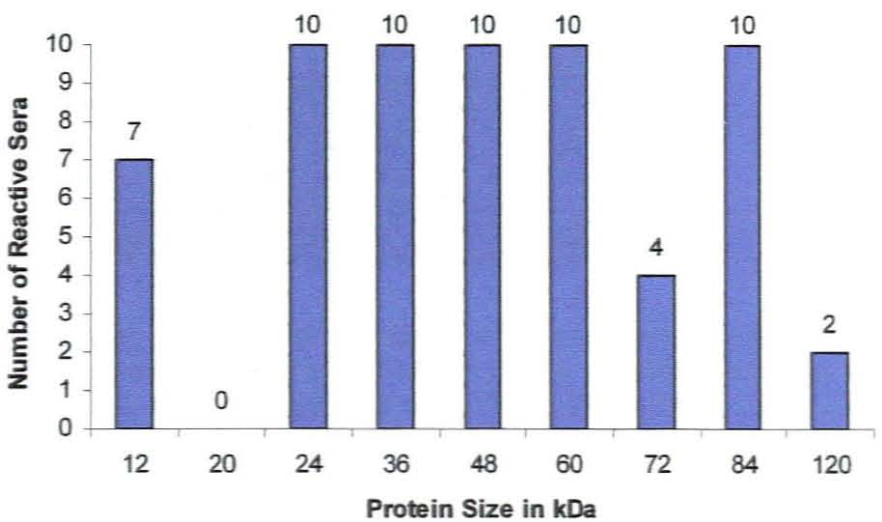


**Figure 4.7. Frequency of fresh pilchard IgG-reactivity.** IgG-binding proteins of the fresh pilchard extract to antibodies of 10 fish-allergic workers was quantified and compared to each other. The protein molecular weight in kDa is shown at the bottom of the graph, while the numbers of reactive sera specimens are indicated on the left-hand side.

### 4.7.2 Reactivity with Anti-Human IgE

IgE binding to fresh pilchard extract proteins was demonstrated by exposing the membrane, containing the fish proteins with sera of 9 fish-allergic workers to this IgE antibody. The molecular weight marker on this membrane ranged from 10 to 250 kDa as shown in Figure 4.8. Clear IgE-reactive bands were seen around 36 kDa, whereas only fainter bands were observed on the rest of the membrane. The most prominent IgE-reactive band on the fresh pilchard protein-containing membrane was the 48 kDa protein-IgE antibody complex.

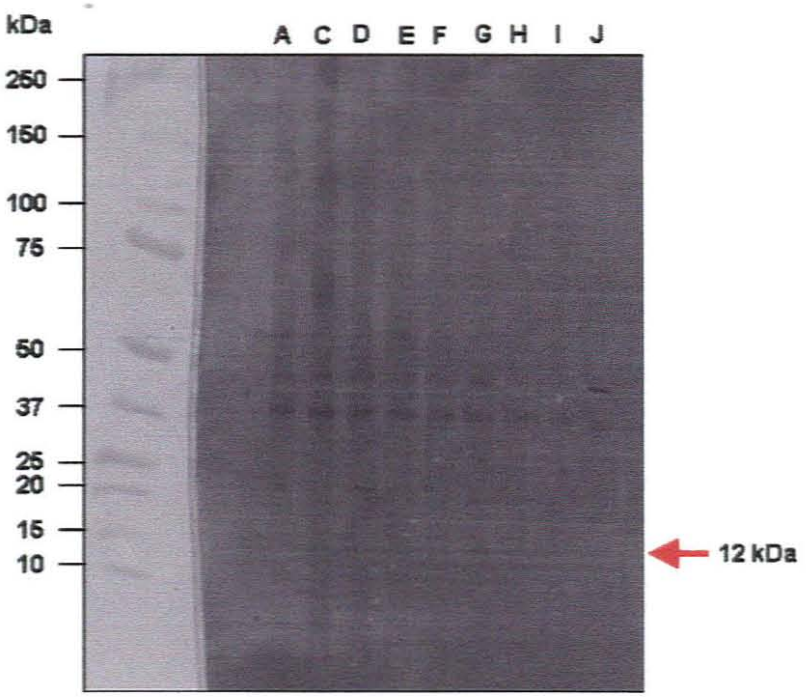


**Figure 4.8. Fresh pilchard IgE-reactivity with sera from fish-allergic workers.** The membrane was exposed to 9 workers' sera and a goat anti-human IgE secondary antibody. Lanes A-J indicate sera from the workers, and the bands on the extreme left represent the molecular weight markers. Serum of worker B was insufficient and not used for this experiment. The position of the 12 kDa protein band, which is the size of parvalbumin, is indicated by the red arrow on the right side of the membrane.

Sera of workers A, C, D, G and J showed IgE-reactivity with a protein in the fresh pilchard extract around 48 kDa in molecular weight, as observed in Table 4.3. Additionally, the 36 kDa protein of the fresh pilchard extract was IgE-reactive to all

workers' sera, a finding which was also illustrated more clearly by Figure 4.9. Only the serum from worker A was IgE-reactive with a 60 kDa protein.

**Table 4.3 Estimated IgE-reactive protein molecular weights in fresh pilchard**

| Molecular Weight in kDa | A | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|
| >250 | - | - | - | - | - | - | - | - | - |
| 250 | - | - | - | - | - | - | - | - | - |
| 150 | - | - | - | - | - | - | - | - | - |
| 120 | - | - | - | - | - | - | - | - | - |
| 85 | - | - | - | - | - | - | - | - | - |
| 55 | X | - | - | - | - | - | - | - | - |
| 48 | X | X | X | - | - | X | - | - | X |
| 37 | X | X | X | X | X | X | X | X | X |
| 35 | - | - | - | - | - | - | - | - | - |
| 25 | - | - | - | - | - | - | - | - | - |
| 20 | - | - | - | - | - | - | - | - | - |
| 12 | - | - | - | - | - | - | - | - | - |
| X = present<br>- = absent | | | | | | | | | |

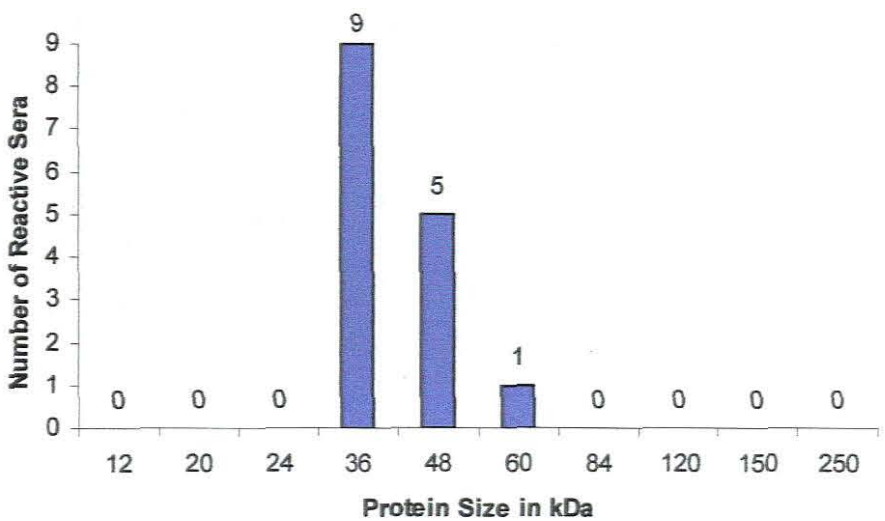


**Figure 4.9. Frequency of fresh pilchard IgE-reactivity.** IgE protein-binding of the fresh pilchard extract proteins to the antibodies of 9 workers was quantified and compared. Protein size in kDa is shown at the bottom of the graph, and the number of reactive sera on the left of the graph.

## 4.8 Frozen Pilchard Anti-Human IgG- and IgE-Reactivity

### 4.8.1 Reactivity with Anti-Human IgG

To demonstrate workers' sera IgG binding to frozen pilchard proteins, the fish proteins were transferred from the SDS-PAGE-gel to a PVDF membrane and probed with 10 fish-allergic workers' sera, using a slot blot apparatus. Broad, prominent reactive bands were observed around 12 kDa, although thinner, also prominent bands could be seen around 48 kDa. Some faint bands were also detected in-between the 12 and 48 kDa bands (Figure 4.10).

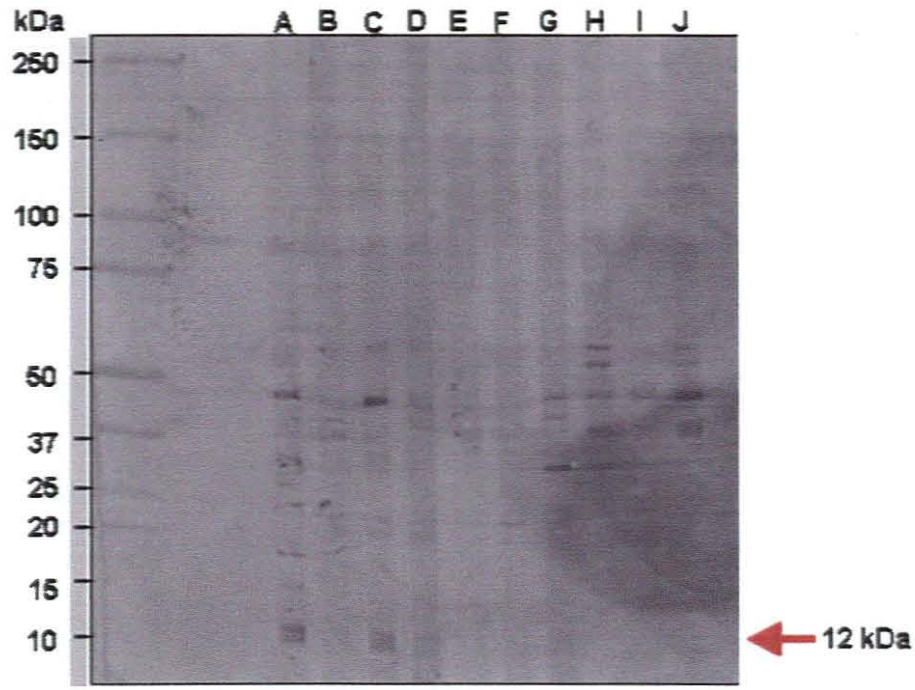


**Figure 4.10. Frozen pilchard IgG-reactivity with fish-allergic workers' sera.** The membrane was exposed to 10 workers' sera and a goat anti-human IgG antibody. Lanes A-J indicate workers' sera and the bands on the extreme left show the molecular weight markers. The red arrow on the right-hand side indicates the molecular weight of parvalbumin at 12 kDa.

Sera of workers A, C and D showed IgG-reactivity to the 12 kDa protein in frozen pilchard. Similar to the graphical depiction in Figure 4.11, Table 4.4 also shows that the 36 kDa protein was bound by 100 % of the IgG-reactive workers' sera, while 7 out of the 10 workers' sera detected the epitopes on the 48 kDa frozen pilchard

protein. The serum from worker A was the most IgG-reactive in general, forming antigen-antibody complexes with proteins at 12, 20, 24, 36, 48, 60 and 84 kDa in molecular weight.

**Table 4.4 Estimated IgG-reactive protein molecular weights in frozen pilchard**

| Molecular Weight in kDa | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| >250 | - | - | - | - | - | - | - | - | - | - |
| 250 | - | - | - | - | - | - | - | - | - | - |
| 150 | - | X | X | X | - | - | - | - | - | - |
| 120 | - | - | - | - | - | - | - | - | - | - |
| 84 | X | X | X | X | - | - | - | - | - | - |
| 60 | X | X | X | X | - | - | - | X | - | X |
| 48 | X | X | - | - | - | X | X | X | X | X |
| 36 | X | X | X | X | X | X | X | X | X | X |
| 24 | X | X | X | X | X | X | X | - | - | - |
| 20 | X | - | - | - | - | - | - | - | - | - |
| 12 | X | - | X | X | - | - | - | - | - | - |
| X = present<br>- = absent | | | | | | | | | | |

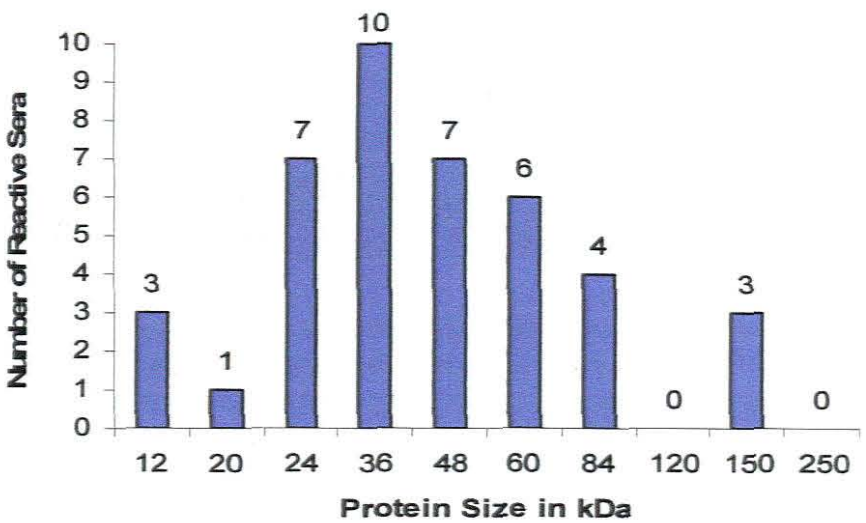


**Figure 4.11. Frequency of frozen pilchard IgG-reactive proteins.** IgG protein-binding of frozen pilchard extract proteins to antibodies of 10 workers was quantified and compared with each other. The protein molecular weights are shown below, ranging from 12 to 250 kDa, and the number of reactive sera on the left of the graph.

### 4.8.2 Reactivity with Anti-Human IgE

The IgE-reactivity of 9 fish-allergic workers' sera to frozen pilchard proteins was determined by probing the fish extract-bound membrane with the relevant sera, using a slot blot apparatus. Prominent, reactive bands were observed around 36 and 48 kDa as seen in Figure 4.12, with the serum of worker J demonstrating the strongest binding of all with the 48 kDa protein. Some faint bands were also observed below 36 kDa as well as above 48 kDa, but not a 12 kDa band.

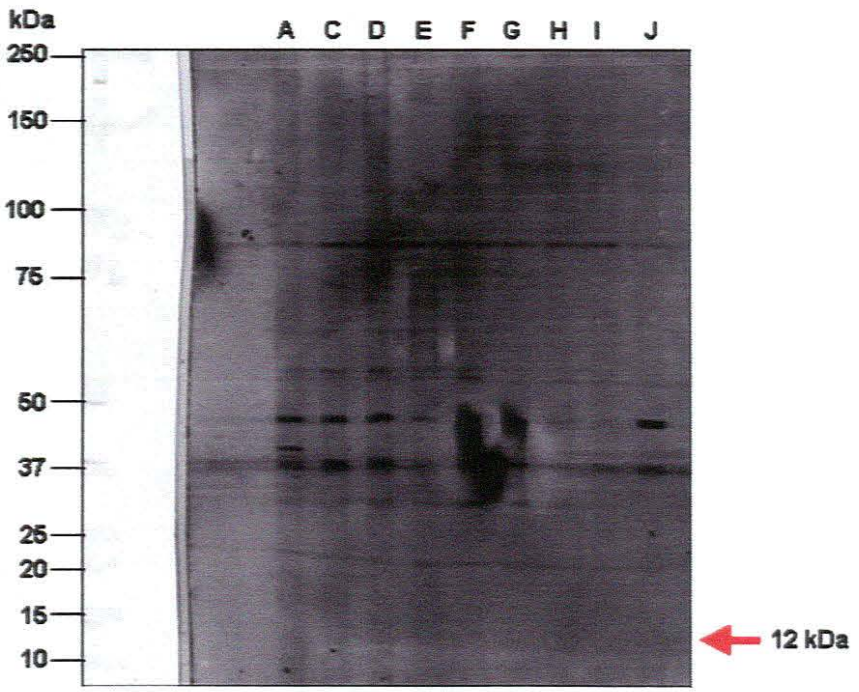


**Figure 4.12. Frozen pilchard IgE-reactivity with sera from fish-allergic workers.** The membrane containing separated proteins from a frozen pilchard extract was exposed to 9 workers' sera and a goat anti-human IgE antibody. The molecular weight markers are shown on the extreme left-hand side of the membrane, and lanes A-J indicate the individual workers' sera. Serum of worker B was insufficient and was not used in this experiment. The position of the 12 kDa protein band, possibly parvalbumin, is indicated on the right-hand side of the membrane by a red arrow.

Sera of workers A, C, D and J were IgE-reactive to the 24 kDa frozen pilchard protein, whereas workers A, C and D were showing the highest IgE-reactivity of the 9 sera in general, demonstrating reactivity with proteins at 24, 36, 48, 60 and 84 kDa (Table 4.5). The sera of workers E, F and G are demonstrating the second highest reactivity, binding to proteins at 36, 48 and 60 kDa. From the graph in Figure 4.13 it

could be seen that all sera are reactive to the protein at 36 and 48 kDa. The 84 kDa protein was bound by the least workers' sera, making it the least recognised protein in the frozen pilchard extract.

**Table 4.5 Estimated IgE-reactive protein molecular weights in frozen pilchard**

| Molecular Weight in kDa | A | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|
| >250 | - | - | - | - | - | - | - | - | - |
| 250 | - | - | - | - | - | - | - | - | - |
| 150 | - | - | - | - | - | - | - | - | - |
| 120 | - | - | - | - | - | - | - | - | - |
| 84 | X | X | X | - | - | - | - | - | - |
| 60 | X | X | X | X | X | X | - | - | - |
| 48 | X | X | X | X | X | X | X | X | X |
| 36 | X | X | X | X | X | X | X | X | X |
| 24 | X | X | X | - | - | - | - | - | X |
| 20 | - | - | - | - | - | - | - | - | - |
| 12 | - | - | - | - | - | - | - | - | - |
| X = present<br>- = absent | | | | | | | | | |

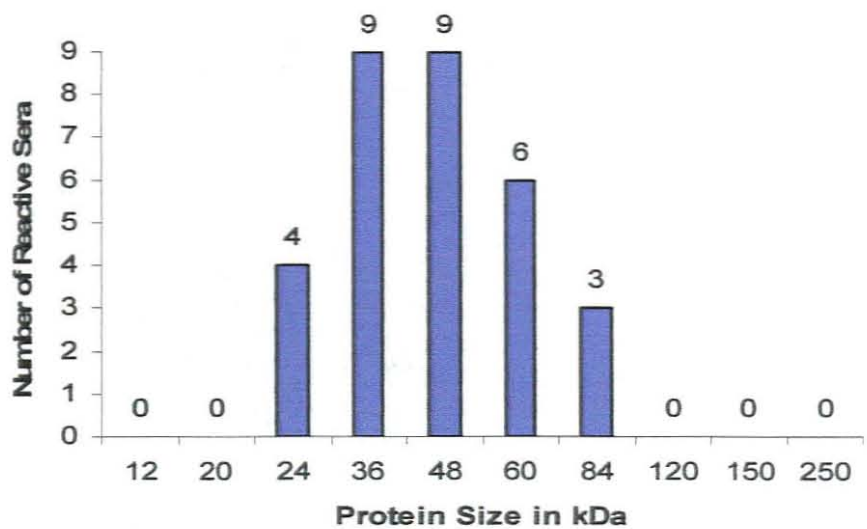


**Figure 4.13. Frequency of frozen pilchard IgE-reactive proteins.** IgE-reactivity of frozen pilchard extract proteins to the sera of 9 workers was quantified and compared to the different protein sizes, which are indicated from 12 to 250 kDa. The number of reactive sera is indicated on the left of the graph.

## 4.9 Canned Pilchard Anti-Human IgG- and IgE-Reactivity

### 4.9.1 Reactivity with Anti-Human IgG

Separated fish proteins were transferred from a SDS-gel onto a PVDF membrane, and exposed to sera of 10 workers with occupational allergies to fish. IgG-dependent reactions between the antibodies of the workers' sera and the membrane-bound fish proteins were observed as seen in Figure 4.14. The molecular weight marker was separated between 10 and 250 kDa, while the IgG-reactive protein bands were detected ranging from around 12 to 250 kDa. The most prominent reactive band was seen in worker G at about 36 kDa, with a less prominent band of the same molecular weight in worker J.

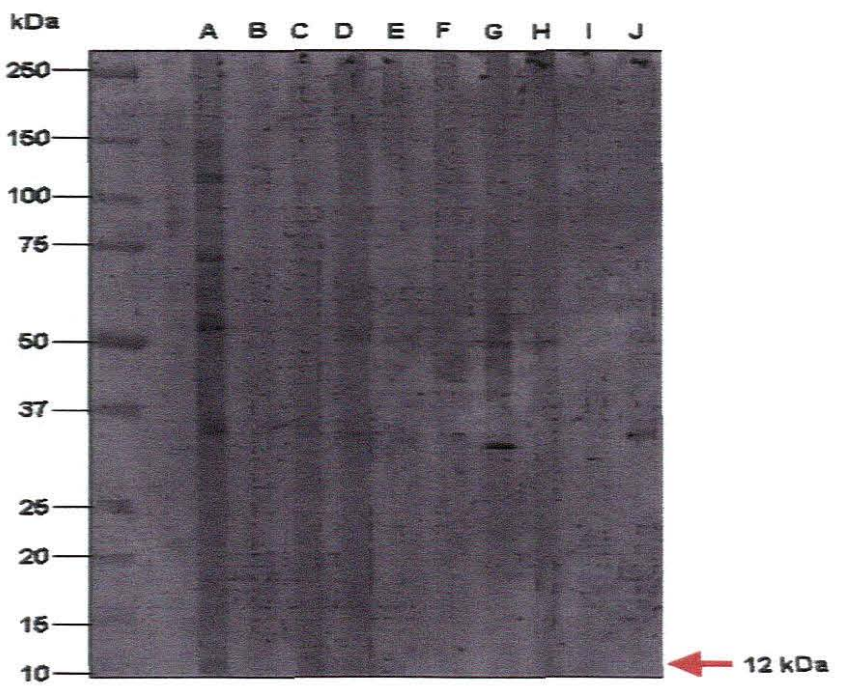


**Figure 4.14. Canned pilchard IgG-reactivity with sera from fish allergic workers.** The membrane with SDS-PAGE separated proteins was exposed to 10 workers' sera before being probed with a goat anti-human IgG antibody. Lanes A-J indicate the sera from the workers, whereas the bands on the extreme left show the molecular weight markers. The red arrow on the right-hand side indicates the molecular size and location of parvalbumin.

Generally, the serum of worker A showed the most IgG-reactive bands amongst the allergic workers in this study, demonstrating binding with proteins around 12, 36, 60, 72 and 108 kDa, as also shown by Table 4.6, which demonstrates the comparative positions of the IgG-reactive protein bands on the membrane in table format. The serum of worker H showed the least reactive bands, detecting one IgG-binding protein at 60 kDa. Most of the sera demonstrated binding with proteins at 36 and 60 kDa. The graph in Figure 4.15 also highlights this finding, illustrating that sera of eight workers are reactive to proteins at 36 and 60 kDa. It also shows that two workers' sera bind to the protein at 12 kDa.

**Table 4.6 Estimated IgG-reactive protein molecular weights in canned pilchard**

| Molecular Weight in kDa | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| >250 | - | - | - | - | - | - | - | - | - | - |
| 250 | - | - | - | - | - | - | - | - | - | - |
| 150 | - | - | - | - | - | - | - | - | - | - |
| 108 | X | - | - | - | - | X | - | - | - | - |
| 84 | - | - | - | - | - | - | - | - | - | - |
| 72 | X | - | X | - | - | - | - | - | - | - |
| 60 | X | X | X | X | X | - | X | X | - | X |
| 48 | - | - | - | - | - | - | - | - | - | - |
| 36 | X | X | X | X | X | X | X | - | - | X |
| 24 | - | - | - | - | - | - | - | - | - | - |
| 20 | - | - | - | - | - | - | - | - | - | - |
| 12 | X | - | X | - | - | - | - | - | - | - |
| X = present<br>- = absent | | | | | | | | | | |

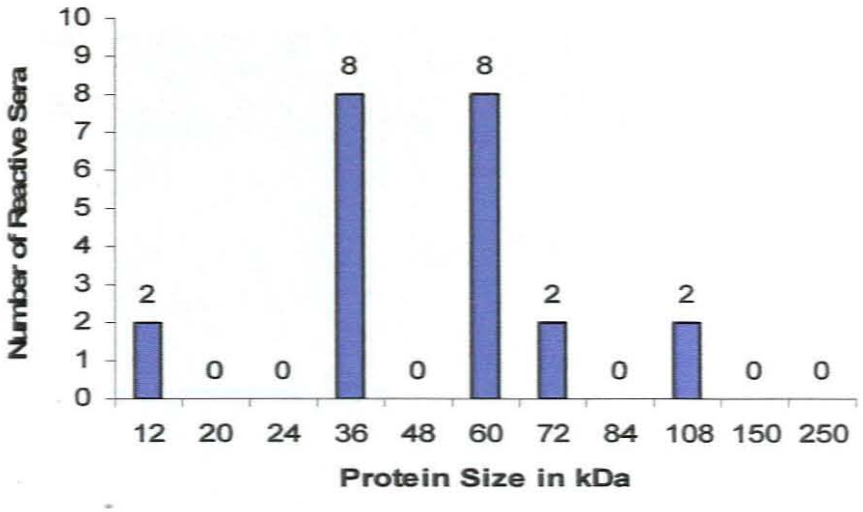


**Figure 4.15. Frequency of canned pilchard IgG-reactive proteins.** Canned pilchard IgG-reactivity to sera from 10 workers was quantified and compared to all possible proteins on the fish extract.

### 4.9.2 Reactivity with Anti-Human IgE

The IgE-reactivity of ten workers' sera with the canned pilchard proteins can be seen in Figure 4.16. The molecular weight marker was separated between 10 and 250 kDa. The most prominent reactive protein in the samples was observed at 60 kDa by the serum of worker J, while the serum of worker A reacted to proteins at 12, 36, 72, 120, and 250 kDa, which is also apparent in Table 4.7. A 60 kDa protein was recognised by the sera of workers E, F, G, H, I and J, whereas the serum of worker D only reacts with a protein around 20 kDa.

The frequency of IgE-reactivity to canned pilchard proteins at 36 and 60 kDa was 5 and 6 workers' sera, respectively (Figure. 4.17). Sera of two workers demonstrated canned pilchard protein-binding at 12 kDa, while proteins at approximately 20 kDa, as well as others at 72, 120 and 250 kDa were detected by one workers' serum each.

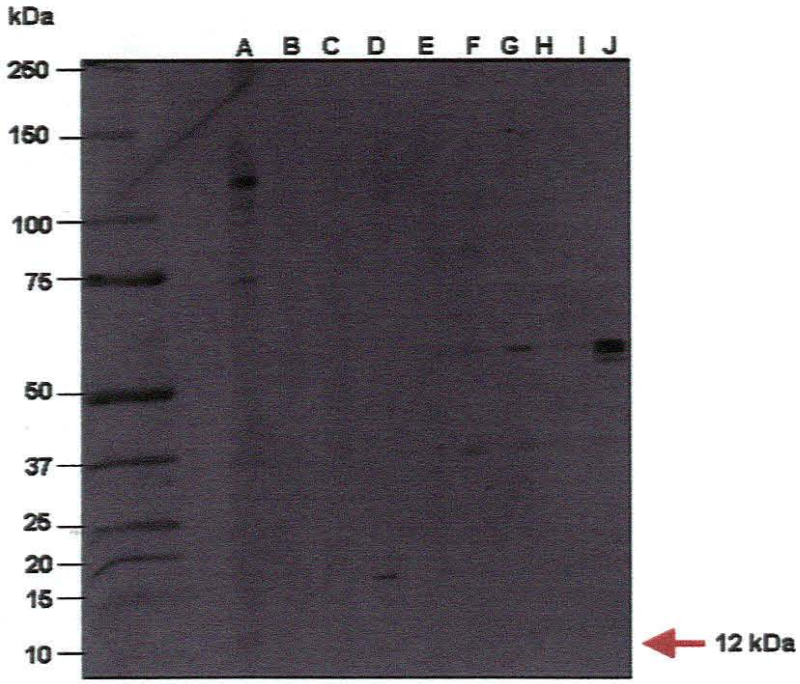


**Figure 4.16. Canned pilchard IgE-reactivity with sera from fish-allergic workers.** Goat anti-human IgE was used as a detecting antibody, after the membrane was probed with the 10 workers' sera. The molecular weight marker is seen on the extreme left hand side, followed by the lanes A-J probed with sera from the individual workers. The arrow on the right-hand side indicates parvalbumin at 12 kDa.

**Table 4.7 Estimated IgE-reactive protein molecular weights in canned pilchard**

| Molecular Weight in kDa | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| >250 | - | - | - | - | - | - | - | - | - | - |
| 250 | X | - | - | - | - | - | - | - | - | - |
| 150 | - | - | - | - | - | - | - | - | - | - |
| 120 | X | - | - | - | - | - | - | - | - | - |
| 84 | - | - | - | - | - | - | - | - | - | - |
| 72 | X | - | - | - | - | - | - | - | - | - |
| 60 | - | - | - | - | X | X | X | X | X | X |
| 48 | - | - | - | - | - | - | - | - | - | - |
| 36 | X | X | X | - | - | X | X | - | - | - |
| 24 | - | - | - | - | - | - | - | - | - | - |
| 20 | - | - | - | X | - | - | - | - | - | - |
| 12 | X | - | X | - | - | - | - | - | - | - |
| X = present<br>- = absent | | | | | | | | | | |

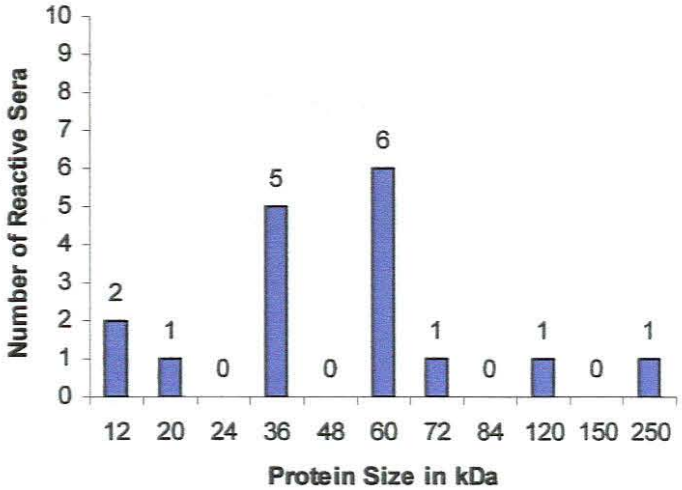


**Figure 4.17. Frequency of canned pilchard IgE-reactive proteins.** The canned pilchard IgE-reactivity to sera from 10 workers is quantified and compared in the different protein sizes, indicated in kDa at the bottom of the graph. The number of reactive sera is shown on the left of the graph.

# Chapter 5

# Discussion

## 5.1 Biochemical and Anti-Parvalbumin Analysis

The protein extraction from fresh, frozen, and canned pilchard, as well as anchovy, tuna, yellow tail, snoek, hake, and pollack was carried out efficiently and the total protein concentration estimated from the extracts ranged from 3.10 mg/ml for canned pilchard to 9.54 mg/ml for frozen pilchard. Here, it can be observed how the total protein concentration in pilchard was changed by different conditions, in particular temperatures that they were exposed to after being caught. The pilchard that was frozen immediately when caught, showed that less proteins were denatured up to the point of collection and processing, whereas the fresh pilchard was just stored at 4°C, showing a significantly lower protein concentration than the frozen pilchard extract. This decrease was probably as a result of protein denaturation. The canned pilchard had the lowest total protein concentration, indicating the extent to which the proteins were denatured by the heat during processing.

On separation of proteins by SDS-PAGE, protein fragments ranging from 10 to 250 kDa in molecular weight were observed. Fresh and frozen pilchard extracts showed the same protein bands to be present after staining with Coomassie Brilliant Blue dye, but all the bands appeared stronger to some degree in frozen pilchard,

confirming the likelihood that the proteins in the frozen extract were better preserved than the proteins in fresh pilchard. Generally, the proteins in the fresh and frozen pilchard extracts ranged from 12 to about 84 kDa, whereas the canned pilchard extract only had the 12 and 36 kDa protein bands in common with fresh and frozen pilchard. The rest of the proteins in the canned pilchard were above 100 kDa in size, probably indicating the effects of heat on these proteins. Heat can either cause unfolding or aggregation of molecules, resulting in decreased exposure of hydrophobic sites and loss of solubility (Nakai and Li-Chan, 1989). The aggregation of the protein molecules might therefore have resulted in larger sized molecules, which were observed as appearing in the higher molecular weight regions. Similar to these results, an experiment by Porcel *et al* (2001) showed that the Coomassie staining of raw and cooked sole and hake extracts after SDS-PAGE separation demonstrated that the two raw fish extracts' protein bands were observed ranging between 26 and 100 kDa, compared to the two cooked fish extracts consisting mainly of higher molecular weight proteins, except for a 28 kDa in cooked hake extract (Porcel *et al.*, 2001).

In this study, the protein band around 12 kDa in the canned pilchard extract emerged more prominent than the 12 kDa bands demonstrated by fresh and frozen pilchard. This finding suggests that the 12 kDa protein is heat stable, and although most proteins were denatured as indicated by the total protein concentration and SDS-PAGE results, the 12 kDa protein band of the canned pilchard extract appeared to be more intense than it was before being processed for canning purposes. The intensity of the 12 kDa protein band in fresh pilchard was weaker than in canned pilchard, but seemed relatively stronger than in frozen pilchard, indicating a possible trend that with higher temperatures, the 12 kDa protein which is the putative parvalbumin in

these experiments, becomes more significant. The rest of the fish extracts showed protein band accumulation between 36 and 60 kDa, a feature that was also apparent in the fresh and frozen pilchard extracts, but not in the canned pilchard extract.

To assess the efficient transfer of protein from a gel to a membrane for the purpose of these experiments, a membrane was stained with Coomasie Brilliant Blue after the transfer process was completed. The stained blot indicated that optimal protein transfer occurred. The transferred proteins of fresh pilchard were separated by SDS-PAGE between 10 and 84 kDa in molecular weight. All the proteins were transferred onto the PVDF membrane, suggesting that the process of transferring proteins from the gels to PVDF membranes, in the subsequent experiments could be accepted as reliable and succeeding immunoblot experiments could be performed.

On the immunoblot of all fish extracts with a commercial monoclonal anti-parvalbumin antibody, a 12 kDa protein was present in all the different types of fish extracts. Judging from the molecular weight size, reactivity to monoclonal anti-parvalbumin, and the relative heat-stability of this 12 kDa protein, it was deduced to be parvalbumin. Parvalbumin belongs to a group of calcium-binding sarcoplasmic proteins (Kretsinger *et al.*, 1991), which are present in high amounts in the white muscle of lower vertebrates (Perchere, 1997), and in low amounts in fast-twitch skeletal muscles of higher vertebrates (Lehky *et al.*, 1974). It has been isolated and identified as a major fish allergen, and its sequence has been determined in Baltic cod (Elsayed and Bennich, 1975), Atlantic salmon (Lindstrom *et al.*, 1996), Carp (Bugajska-Schretter *et al.*, 2000), Atlantic cod (Das Dores *et al.*, 2002), mackerel (Hamada *et al.*, 2003), and most recently in Alaska pollack (Van Do *et al.*, 2005).

Following immunoblotting with anti-parvalbumin, tuna and snoek showed very faint parvalbumin bands. This observable fact was first reported in 1992 by Bernhisel-Broadbent and colleagues, who noticed the absence of parvalbumin in tuna during their study on in vitro and oral challenges in fish-allergic patients (Bernhisel-Broadbent *et al.*, 1992a). Thereafter, another group reaffirmed the absence of parvalbumin in tuna during their study (James *et al.*, 1997). Contradicting these two groups, a study to identify and characterise allergens in tuna showed that parvalbumin is present in tuna (Yamada *et al.*, 1999). Verifying this fact, scientists from Singapore confirmed the presence of parvalbumin in tuna (*Thunnus tonggol*) and observed that parvalbumin was present in the white muscle of the fish, but not in the red muscle. They concluded that the possibility of including a substantial red muscle portion for analysis can result in the absence of parvalbumin in tuna (Lim *et al.*, 2005). More recently, the allergenicity and allergens present in different fish muscles were compared. White and dark muscle extracts were examined for reactivity with IgE in fish-allergic patients using ELISA, and immunoblotting for allergen content. These experiments concluded that the fish dark muscle was less allergenic than the white muscle because parvalbumin was contained in much lower levels in the dark muscle compared to the white muscle (Kobayashi A, 2006). Since the tuna from this study was obtained in fillet form, the possibility is high that there was a considerable amount of red muscle present. Therefore it will be advisable to collect whole fish specimens and specifically sample the white muscle for the purposes of allergenicity studies.

The presence of multiple bands of 12 kDa depicted the presence of possible oligomeric forms of parvalbumin present in these fish extracts. Dimeric, trimeric, and

tetrameric forms of parvalbumin were most probably detected in frozen pilchard and anchovy, at 24, 36, and 48 kDa, respectively, whereas tuna and yellow tail extracts only showed the 36 and 48 kDa without the 24 kDa in between. This oligomerisation of fish parvalbumin has been reported for the first time by Das Dores *et al.* in 2002 during their characterisation of a parvalbumin allergen of Atlantic cod (Gad m 1). Prior to that report, such an oligomerisation has only been observed in an avian thymic parvalbumin in the absence of reducing agents (Henzl *et al.*, 1995). Additionally, a protein in the region of 60 kDa was detected in the frozen pilchard, anchovy, tuna, and yellow tail extracts, with the frozen pilchard extract demonstrating binding to proteins around 60 and 84 kDa, respectively.

Therefore, similar to the findings by Das Dores in their characterisation of Gad m 1, it may be suggested that the reducing conditions in the experiments in this study failed to dissociate the oligomers completely. The conditions used for the purpose of these experiments were optimised using the fresh pilchard extract and this extract demonstrated strong binding to a protein around 12 kDa.

For consistency purposes, all fish extracts should be treated similarly, by considering the amount of protein loaded on to gels, dilution with sample buffers, as well as primary and secondary antibodies. Moreover, to ensure proper dissociation of oligomers, the sample buffer preparations should be adjusted according to each of the extracts to be separated electrophoretically.

## 5.2 Immunological Analysis using Sera of Sensitised Rabbits

On initial immunoblotting experiments with the two antisera on canned pilchard and fresh anchovy extracts, the anti-pilchard serum demonstrated strong binding to pilchard proteins in the regions of 12 and 14 kDa, as well as weaker bands between 36 and 60 kDa. Binding to only a 12 kDa protein was observed in the anchovy extract by the anti-pilchard serum. The anti-anchovy serum showed similar size proteins in the pilchard extract as the anti-pilchard serum, but demonstrated stronger binding with a protein at about 12 kDa, as well as proteins in the range of 28-48 kDa. The 12 kDa proteins recognised on both extracts were confirmed to be parvalbumin (Lopata *et al.*, 2005).

With the exception of the tuna extract, the polyclonal rabbit anti-pilchard antibody demonstrated binding to a 12 kDa protein. Predictably, the frozen pilchard extract, together with the anchovy and yellow tail extracts, showed the strongest binding. The 12 kDa protein binding showed the presence of parvalbumin cross-reactivity in different types of fish. The frozen pilchard extract demonstrated stronger binding to the rabbit anti-pilchard antibody than the fresh and canned pilchard extracts, because the proteins in the frozen pilchard extract were theoretically better preserved when the pilchard was frozen soon after being caught. In fish that was frozen immediately and stored at subzero temperatures, the bacterial growth was inhibited, and both enzyme and chemical actions were inhibited. The longer it was left unfrozen after catching, the more changes occurred in the proximate composition of the fish (Licciardello, 1990). Therefore, the amino acid sequence and secondary structures of the frozen pilchard proteins, in comparison to the fresh and canned pilchard proteins, most probably resembled the proteins used to sensitise the rabbits more closely.

Only the 12 kDa protein was recognised by this serum in the canned pilchard extract, whereas Lopata *et al* (2005) demonstrated binding to weaker bands in the regions between 35 and 55 kDa. Another observation that differs from previous reports is that additional weaker bands than the 12 kDa protein were detected around 24, 36, and 48 kDa in the anchovy extract. These findings, together with fresh and frozen pilchard, tuna, yellow tail and snoek sharing the same reactivities around 24, 36, and 48 kDa, strongly indicated the presence of dimeric, trimeric and tetrameric forms of parvalbumin. Assuming this, hake and pollack only demonstrated dimeric forms of parvalbumin, which was observed at 24 kDa. The higher molecular weight proteins detected in tuna and yellow tail could also indicate oligomeric forms of parvalbumin, as a result of insufficient dissociation of the proteins.

As expected, the rabbit anti-anchovy bound prominently to the anchovy extract with a 12 kDa protein. Again, cross-reactivity of parvalbumin to different types of fish was demonstrated by the 12 kDa proteins being recognised in all the extracts. Tuna and snoek showed the weakest bands at around the 12 kDa region, but together with anchovy and yellow tail, bands which appear to most likely be weakly dissociated parvalbumin oligomers, protein binding around 24, 36, and 48 kDa were observed. Contrary to the finding by Lopata *et al* (2005), where the rabbit anti-anchovy antisera detected proteins in the range of 35-55 kDa in canned pilchard in addition to the 12 kDa protein, the canned pilchard extract only demonstrated a 12 kDa binding in this study. Fresh pilchard, hake and pollack extracts also showed binding to the 12 kDa protein exclusively.

## 5.3 Immunological Analysis using Sera of Sensitised Workers

When immunoblotted, the fresh pilchard extracts demonstrated IgG- and IgE-reacting proteins ranging from 12 to 84 kDa in molecular weight, while the frozen pilchard extract displayed reactive proteins ranging between 12 and 150 kDa. The canned pilchard extracts' reactive proteins ranged from 12 to 250 kDa. As seen in the SDS-PAGE experiments, these proteins were present in the relevant extracts following staining gels with Coomassie blue stain. Therefore, the reactivity displayed can either be ascribed to novel proteins not previously proven to be allergenic or the possibility of parvalbumin oligomerisation and present in a ladder of dimers, trimers tetramers and so forth. The latter is the most probable option, as the dissociating conditions of these experiments may have been inadequate as discussed previously.

Similarly, Hilger *et al* (2004) also observed oligomeric forms of parvalbumin while investigating fish allergic patients' IgE antibody cross-reactivity with frog parvalbumin. The formation of these additional immuno-reactive bands was attributed to polymer or aggregate formations and/or a possible complexing of parvalbumin with other proteins.

Another group suggested that fish allergenicity can be altered as a result of processing. They examined the allergens in canned tuna and salmon using SDS-PAGE, immunoblotting and ELISA inhibition experiments, and compared the protein fractions and allergenicity of the extracts to the proteins in raw and cooked tuna and salmon. The SDS-PAGE revealed a significant loss of definable protein fractions in the canned fish extracts compared to raw and cooked fish extracts, whereas the

immunoblot analysis showed minimal IgE-specific binding to canned fish extracts, contrasting the raw and cooked fish extracts (Bernhisel-Broadbent *et al.*, 1992b).

An additionally possible explanation for the increase in the formation of oligomeric forms of allergenic proteins can be drawn from experiments conducted by Dory *et al* (1998). They investigated the probable causes of the presence of an extensive range of IgE-reactive proteins in a cod extract during immunoblotting procedures and detected proteins ranging from 12-130 kDa, which they suspected to be aggregates of the 12 kDa parvalbumin. These bands were also detected by a monoclonal anti-parvalbumin antibody to further suggest their origin to be from this 12 kDa protein. In particular, an 18 kDa band was detected only in fish stored for several days, but absent in fresh samples, and there was a general increase in the relative amounts of 41, 80, 104 and 130 kDa proteins noted with longer storage periods. This group concluded that the storage conditions may influence the relative distribution of IgE-reactive protein bands in fish extracts. In accordance with the findings from this group, it was demonstrated here that, additional higher molecular weight allergens were seen in frozen pilchard extracts' IgE-reactivity experiments as compared to fresh pilchard extracts' IgE-reactivity (Dory *et al.*, 1998).

Immunoblotting of fresh pilchard extract with anti-human IgG demonstrated a wider range of binding to different proteins compared to the immunoblotting with anti-human IgE. Additionally, the proteins which were recognised by both anti-human IgG and IgE antibodies, namely at 36, 48 and 60 kDa were more readily reactive with the anti-human IgG antibody. No IgE reactivity of proteins at around 12 kDa was observed, whereas the anti-human IgG detected proteins at that size in sera of 4 out of

10 workers. At 36 kDa, all 10 workers' sera demonstrated IgG-binding and 9 out of 9 workers' sera detected IgE-binding at 36 kDa as well. Again, only 5 out of the 9 workers' sera were observed to bind with IgE at about 48 kDa, while all 10 sera detected IgG-binding. IgE binding was further observed at about 60 kDa by the serum from 1 worker out of a possible 9, compared to 10 out of 10 workers' sera by the anti-human IgG antibody.

Similarly, the IgE-reactivity to the frozen pilchard proteins was reduced to only 5 proteins demonstrating IgE-reactivity, compared to 8 different proteins, ranging from 12-150 kDa detecting IgG-binding of the workers' sera. In contrast to the reactivity patterns seen in fresh pilchard extracts, the frequency of IgE-binding of the frozen pilchard proteins detected in common by both the anti-human IgG and IgE antibodies that were at 24, 36, 48 and 84 kDa, remained the same compared to the frequency of IgG-reactivity in the same extract. The frequency of the 60 kDa protein's IgE-reactivity was equal when compared to the IgG-reactivity.

Contrary to the fresh and frozen pilchard extracts' IgG- and IgE-binding patterns, the canned pilchard IgE-reactive proteins were more than the IgG-binding ones. A 20 kDa protein was reactive with the anti-human IgE antibody, but not reactive with the anti-human IgG antibody. This protein was also not reactive in both the fresh and frozen pilchard extracts, neither with the anti-human IgG nor with the anti-human IgE antibodies. Otherwise, the frequency of IgG- and IgE-binding of canned pilchard proteins shared the same pattern as the fresh and frozen pilchard proteins, with less IgE-reactivity prevalence in all commonly detected proteins compared to the IgG-reactivity.

Furthermore, IgG antibodies could not be correlated to patient symptoms in previous studies. A study by McSharry and Wilkinson (1987), where they compared serum IgG and IgE antibodies against aerosolised antigens from seafood processing workers, serum IgG antibody was detected with equal frequency and titre in symptomatic and asymptomatic workers. Prior to that, another study, estimating IgG, IgA and IgE antibodies in children with food allergies and atopic dermatitis, showed that IgG antibody titres to cow's milk, egg-white, fish, soy-bean and green peas seemed to parallel the IgE antibody titres in all those food antigens, but it was again impossible to correlate the IgG antibody titres to the symptoms which were presented by the children (Dannaeus *et al.*, 1977).

Unfortunately, these immublotting experiments were conducted without negative control sera. The inclusion of negative controls would have indicated whether the allergenic proteins in the experiments were specifically detected by antibodies used, or would have clarified whether binding was as result of non-specific cross-reactivity or contamination. However, Table 3.1 in fact confirms that all workers' sera tested prior to immunoblotting experiments were sensitive to either pilchard or anchovy by means of a fish IgE-specific CAP-RAST analysis and/or SPT with extracts from these respective fishes.

# Chapter 6

# Conclusion and Future Experiments

In this study, fish proteins were extracted and their concentrations determined. It was observed that the total protein concentrations in pilchard were decreasing with an increase in storage and processing temperatures.

A putative parvalbumin candidate protein, 12 kDa in size, was detected in the denaturing polyacrylamide gels, monoclonal anti-parvalbumin and polyclonal rabbit anti-sera immunoblots in all fish protein extracts. Oligomeric forms of this 12 kDa protein were observed throughout these immunologic analyses, frequently occurring in dimeric, trimeric and tetrameric states.

The IgG- and IgE-reactivities of the sensitised workers' sera to the fish protein extracts were compared in fresh, frozen and canned pilchard. The effects of storage and processing temperatures on these three pilchard extracts could be correlated with the different reactivity profiles of the workers' sera to the fish proteins. IgG-reactivity was generally more prevalent than IgE-reactivity; the oligomeric aggregates of the 12 kDa protein were frequently detected with the sensitised workers' sera. Moreover, the IgG-and IgE-reactivities of the canned pilchard extract showed proteins of higher molecular weights to be more reactive than that of fresh and frozen pilchard extracts, while the latter extracts are demonstrating preferential antibody binding to lower molecular weight proteins.

To corroborate the identity of the 12 kDa antigenic protein to be parvalbumin, molecular analyses will have to be performed, whereby the 12 kDa protein will have to be isolated, purified and subjected to amino acid sequencing. This will facilitate comparison of this putative parvalbumin amino acid sequence with known allergenic protein sequences. Furthermore, the supposed oligomeric forms could also be sequenced to establish whether they are indeed of parvalbumin origin or whether they constitute novel protein allergens.

Additionally, air sampling in pilchard processing factories could be performed and the proteins extracted from the filters. Development of techniques for the isolation and purification of these airborne proteins will enable sequencing and thus identification of reactive proteins in the air sample protein extracts. This approach may substantiate the occurrence of known allergens in air samples or it may enable the identification of novel allergens in air samples in these fish processing industries.

# Appendix

## 1.1 Phosphate Buffered Saline (10x)

1.37 M NaCl

0.03 M KCl

0.01 M $Na_2HPO_4$

Make up to 1L with distilled water. Prepare 1x PBS and store at room temperature.

## 1.2 Sealing agar

1% agarose in distilled water. Boil solution until agarose completely dissolves in the water.

Store at room temperature.

## 1.3 Separating/Running Gel Buffer (2.5x)

1.875M Tris-Cl

0.25% SDS

Adjust pH to 8.9 with HCl and store at room temperature.

## 1.4 Ammonium Persulphate (10%)

10µg of Ammonium Persulphate dissolved in 100µl of distilled water. Store at -20°C.

## 1.5 Electrophoresis Buffer (5x)

0.5M Tris

1.92M glycine

0.5% SDS

pH should be 8.8 without adjustment. 1x solution done by diluting with distilled water. Store at room temperature.

## 1.6 Coomassie Stain

0.1% Coomassie Brilliant Blue dissolved in destain solution.

Store at room temperature.

## 1.7 SDS-PAGE Sample Buffer (5x)

0.225M Tris-Cl, pH 6.8

50% glycerol

5% SDS

0.05% bromophenol blue

0.25M dithiothreitol (DTT)

Make 50µl aliquots and store at -20°C.

## 1.8 Destaining solution

15% methanol

7.5% acetic acid

Make up to 1L with distilled water, cover with aluminium foil, and store at room temperature.

## 1.9 Stacking Gel Buffer (5x)

0.3 M Tris-phosphate

0.5% SDS

Adjust pH to 6.7 with phosphoric acid and store at room temperature.

## 1.10 Transfer Buffer (10x)

20.8 mM Tris

1.6 M Glycine

Add up to 1L with $dH_2O$ and store at room temperature.

Make up 1x Transfer buffer by adding 700 mL $dH_2O$ to 100 ml 10x Tranfer buffer and 200 mL methanol.

## 1.11 Blocking Buffer

5% milk powder in 1xTBS.

Store at 4°C.

## 1.12 Tris-Buffered Saline (10x)

100 Mm Tris-Cl

0.9% NaCl

Adjust pH to 7.4, prepare a 1x TBS by diluting with distilled water, and store at room temperature.

## 1.13 Washing Buffer

1% milk powder

0.05% Tween 20

Make up to 1L with 1xTBS and store at 4°C.

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