Characterization of a 36 Kda Fish Protein and Its Application to the Development of an Immunoassay for the Detection of Fish Muscle

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CHARACTERIZATION OF A 36 KDA FISH PROTEIN AND ITS APPLICATION TO THE DEVELOPMENT OF AN IMMUNOASSAY FOR THE DETECTION OF FISH MUSCLE

By

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# Table of Contents

List of Tables ........................................................................................................................................ v

List of Figures ......................................................................................................................................... vi

Abstract .................................................................................................................................................. vii

Introduction ............................................................................................................................................ 1

1. Background ...................................................................................................................................... 1

2. Hypotheses ...................................................................................................................................... 4

3. Objectives ...................................................................................................................................... 5

CHAPTER ONE ...................................................................................................................................... 6

LITERATURE REVIEW ...................................................................................................................... 6

1.1 Importance of developing a fish immunoassay ............................................................................. 6

1.1.1 Fish allergy and its associated regulations for consumer protection .................................... 6

1.1.2 Concerns regarding the use of fishmeal in animal feed ......................................................... 8

1.2 Currently available methods for the detection of fish in food .................................................... 9

1.3 Thermal-stable proteins in fish muscle ......................................................................................... 12

1.4 The characteristics of tropomyosin ............................................................................................ 14

1.4.1 Fish tropomyosin isoforms and distribution .......................................................................... 15

1.4.2 The allergenicity of tropomyosin .......................................................................................... 16

1.4.3 Thermal-stability of tropomyosin ......................................................................................... 17

1.4.4 The molecular weight of tropomyosin ............................................................................... 18

1.4.5 The structure of tropomyosin ............................................................................................... 19

1.5 Potential fish-specific regions of the fish tropomyosin amino acid sequence ............................ 20

1.6 The effect of heat treatment on the epitope. ............................................................................... 20

CHAPTER TWO .................................................................................................................................... 22

CHARACTERIZATION OF A 36 KDA THERMAL-STABLE ANTIGENIC FISH MUSCLE PROTEIN AS A POTENTIAL FISH MARKER PROTEIN ......................................................... 22

2.1 Introduction ................................................................................................................................. 22
2.2 Materials and Methods..................................................................................................... 23
  2.2.1 Materials ................................................................................................................ 23
  2.2.2 Methods ................................................................................................................ 24
    2.2.2.1 Fish sample extraction ............................................................................... 24
    2.2.2.2 Purification of fish 36 kDa protein ............................................................ 24
    2.2.2.3 Purification of fish tropomyosin ............................................................... 25
    2.2.2.4 Amino acid analysis .................................................................................. 25
    2.2.2.5 Trypsin digestion and protein sequence analysis ...................................... 26
    2.2.2.6 Gel electrophoresis and Immunoblot ........................................................ 26
    2.2.2.7 Peptide array for epitope identification ..................................................... 27
    2.2.2.8 Comparison of amino acid sequence between fish and non-fish species.. 27
  2.3 Results and Discussion .................................................................................................... 28
    2.3.1 The thermal stable protein banding pattern in cooked fish samples............... 28
    2.3.2 Molecular weight migration of the 36 kDa protein in the presence of urea ....... 30
    2.3.3 Comparison of amino acid composition and sequence between the 36 kDa protein and fish tropomyosin ................................................................. 31
    2.3.4 The fish-specific region of the 36 kDa protein ...................................................... 32
    2.3.5 The 36 kDa thermal-stable protein as a suitable marker protein for fish detection 32
  2.4 Summary .......................................................................................................................... 33

CHAPTER THREE ...................................................................................................................... 34
DEVELOPMENT OF A SANDWICH ELISA FOR THE DETECTION OF FISH MUSCLE .. 34
  3.1 Introduction ...................................................................................................................... 34
  3.2 Materials and Methods ..................................................................................................... 35
    3.2.1 Materials ................................................................................................................ 35
    3.2.2 Methods ................................................................................................................ 36
      3.2.2.1 Production of polyclonal antibody for fish detection................................. 36
BIOGRAPHICAL SKETCH ................................................................. 96
LIST OF TABLES

Table 1 Optimization of the capture and detection antibodies of the sandwich ELISA..............51
Table 2 The inter-and intra- CV of the sandwich ELISA for raw spiked samples....................52
Table 3 The inter-and intra-CV of the sandwich ELISA for cooked spiked samples. .................53
Table 4 The false-positive and false-negative rates of the sandwich ELISA. ...............................54
LIST OF FIGURES

Fig. 1 Immunoreactivity of MAb 8F5 by indirect ELISA with cooked samples from fish, shellfish, land animals and food additives. .................................................................55

Fig. 2 SDS-PAGE (A) and immunoblot (B) analyses of the antigenic protein of MAb 8F5 in cooked fish samples. ..................................................................................56

Fig. 3 SDS-PAGE analysis of the protein profile of raw (A) and cooked (B) fish samples. .........................................................................................................................57

Fig. 4 Comparison of SDS-PAGE analysis of salmon tropomyosin and cooked fish samples in the absence (A) and presence (B) of urea in the gel. ...........................................58

Fig. 5 Comparison of immunoblot analysis of salmon tropomyosin and cooked fish samples using MAb 8F5 in the absence (A) and presence (B) of urea in the gel. .....................59

Fig. 6 SDS-PAGE (A) and immunoblot (B) analyses using MAb 8F5 with purified salmon 36 kDa protein and purified salmon tropomyosin. ...........................................................60

Fig. 7 Comparison the amino acid composition profiles of the purified Atlantic salmon 36 kDa protein and the purified Atlantic salmon fish tropomyosin ........................................61

Fig. 8 The protein sequence of the tryptic digested fragment of the 36 kDa protein ....................62

Fig. 9 Determination of the amino acid sequence of the MAb 8F5 epitope using a peptide array63

Fig. 10 Immunoreactivity of PAb B by indirect ELISA with raw and cooked samples from fish, shellfish, land animals and food additives. .................................................................64

Fig. 11 Immunoreactivity of PAb BD by indirect ELISA with cooked samples from fish, shellfish, land animals and food additives. .................................................................65

Fig. 12 SDS-PAGE (A) and immunoblot (B) analyses of cooked samples and fish tropomyosin using PAb BD. ........................................................................................................66

Fig. 13 Schematic representation of sandwich ELISA. .............................................................67

Fig. 14 Selection of the antibodies for the development of the sandwich ELISA.........................68

Fig. 15 Optimization of raw (A) and cooked (B) sample dilutions of the sandwich ELISA ....69
Fig. 16 Immunoreactivity of the sandwich ELISA with raw samples from fish, shellfish, land
animals and food additives.................................................................70

Fig. 17 Immunoreactivity of the sandwich ELISA with cooked samples from fish, shellfish, land
animals and food additives........................................................................................................................................71

Fig. 18 Immunoreactivity of the sandwich ELISA with salted fish samples..........................72

Fig. 19 Immunoreactivity of the sandwich ELISA with smoked fish samples. ..................73

Fig. 20 Immunoreactivity of the sandwich ELISA with canned fish samples and fishmeal. ....74

Fig. 21 Limit of detection (LOD) of fish (pollock, whiting, basa) in crab using the sandwich
ELISA. ..................................................................................................................75
ABSTRACT

Fish is classed as a major allergenic food under the Food Allergen Labeling and Consumer Protection Act (FALCPA), which requires accurate information on food allergens to be included in the label of food products. Fishmeal, a fish by-product, is a common protein source in animal feed but it has been banned for use in ruminant feed under the transmissible spongiform encephalopathy regulations in European Union, Japan and Australia. Currently, there is no convenient and reliable method available for the detection of fish in food and animal feed. The overall goal of this study was, therefore, to develop a sandwich enzyme linked immunosorbent assay (sELISA) for the detection of fish and fish products. The specific objectives were to: 1) identify a suitable marker protein to indicate the presence of fish in food; and 2) develop an immunoassay based on this marker protein for the detection of fish in foods.

A 36 kDa thermal-stable protein recognized by a previously developed fish-specific monoclonal antibody (MAb) 8F5 in cooked fish extract was present in all 55 fish species tested. Because of its presence in common food fish species and the thermal-stability of the 36 kDa protein, this was identified as a potentially suitable marker protein. The 36 kDa protein was further characterized along with fish tropomyosin because of their similarity in molecular weight, protein banding pattern and thermal-stability.

The results showed that the 36 kDa protein and fish tropomyosin both exhibited molecular migration in urea gel and were recognized by MAb 8F5 in an immunoblot test. In addition, the 36 kDa protein and fish tropomyosin had matching amino acid compositions and identical protein sequence (12 amino acid residues). Furthermore, the MAb 8F5 epitope contained the conserved region of fish tropomyosin. Based on these results, the 36 kDa was verified to be fish tropomyosin, a ubiquitous muscle protein with equal distribution in muscle tissue in different locations. Fish tropomyosin was therefore deemed a suitable marker protein for fish detection in an immunoassay.

In order to develop a user-friendly sELISA for fish detection, a polyclonal antibody (PAb) was raised against fish tropomyosin to pair with MAb 8F5. However, PAb competes with MAb 8F5 for the same epitope, so PAb was used as both the capture antibody and the detection antibody for the sELISA after depleting the non-specific cross-reactivity. The optimized assay
was specific to both raw and cooked samples of all 64 fish species tested with no cross-reaction with shellfish, land animals and food additives. The assay was also capable of detecting fishmeal and processed fish products (salting, smoking, canning). The assay exhibited low intra- (%CV ≤ 8.9%) and inter-assay variability (%CV ≤ 9.4%). The limit of detection of the assay was 0.1 ppm for both raw and cooked fish (pollock and basa) in crab meat.

The polyclonal antibody-based sELISA developed for this study is expected to be a useful tool for the qualitative detection of fish muscle protein in food products to reduce the risks associated with fish allergies and enforce the food safety laws.
INTRODUCTION

1. Background

Fish has been identified as one of the eight major allergenic foods (fish, shellfish, milk, eggs, peanuts, tree nuts, wheat, and soy) that cause 90% of all food allergenic reactions. The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) requires that all food or products containing proteins from these eight allergenic foods must be declared in the ingredient statement (Public Law 108-282). According to a nationwide survey, 0.4% of the total population suffers from a fish allergy in the United States (Sicherer and others 2004). Because no specific cures are available for food allergy, sensitized consumers rely on accurate food labeling to avoid allergenic foods or their derived ingredients in food. Accurate food labeling is thus vital for reducing the risk of an allergic reaction for sensitized consumers. Reliable analytical assays for specific allergenic foods are very important for enforcing the food labeling laws and assays for the detection of all the major allergenic foods are available except for fish.

The safety issues related to fish usage are not limited to concerns regarding human food but also extend to animal feed. Fishmeal, a common ingredient in animal feed, is banned for use in ruminant feed in European and other countries such as Japan and Australia under transmissible spongiform encephalopathy regulations. Fishmeal is a commercial fish by-product that is commonly used as an ingredient in animal feed. It mainly consists of whole fish or recycled trimmings from fish processing. To prevent the transmission of bovine spongiform encephalopathy, many kinds of animal proteins have been prohibited in feed for food animals, although there is no evidence to indicate that fish meal can transmit bovine spongiform encephalopathy. However, species identification of animal materials in feed is very difficult. Because grinding, drying and rendering are common processes in feed production, most species-specific materials like DNA (deoxyribonucleic acid) and proteins are degraded or denatured during processing. At present, microscopy is the only official method for fish detection in the European Union. However, microscopy is a time-consuming and inconvenient method for routine screening analysis. For the above reasons, the development of a rapid and reliable method for fish detection in both human food and animal feed is urgently needed for both food safety and livestock health.
Currently, DNA-based and protein-based techniques are the major methods for allergenic food detection. However, the advantage of DNA-based techniques relies on the relative thermal-stability of DNA and DNA is not absolutely stable under all food processing conditions. Severe heat treatment like deep frying is known to cause degradation of DNA (Hsieh and others 2004). Furthermore, DNA-based analysis is generally expensive, time-consuming and complex to perform, so DNA-based analysis is not suitable for the routine analysis of large numbers of samples on daily basis. An alternative is to utilize immunoassays, which use antibodies to recognize a specific region (epitope) on a target protein in samples. The marker protein can be a food allergic protein or a marker protein that represents the presence of the allergic food. Immunoassays are commonly used in food industries, laboratories, and governmental agencies for food allergen detection. The two formats of immunoassay, ELISA and immunochromatographic assay (also known as lateral-flow), both provide rapid, sensitive and convenient ways to screen large numbers of samples for routine analysis by professional users or consumers. There are several formats of ELISA, including sandwich ELISA, direct ELISA, indirect ELISA, and competitive ELISA. The format chosen for this study, sandwich ELISA, requires a pair of antibodies that recognize different epitopes on the target protein. In the sandwich ELISA format, the antibody is coated on a solid phase to serve as the “capture antibody” for target proteins in the sample. Another antibody conjugated with chemicals for signal generation is then added as the “detection antibody” to react with the captured target proteins. The major advantage of sandwich ELISA is that the samples do not need further purification prior to use. Compared to other formats of ELISA, this saves time for sample preparation and is a very user-friendly method. Commercial immunoassay kits can be easily utilized and do not require complex equipment, hazardous chemical reagents, or highly-trained personnel.

Existing fish analyses include DNA-based techniques (Lockley and Bardsley 2000), isoelectrofocusing (Hsieh and others 1997; Helrich 1990) and immunoassay (Taylor and Jones 1992; Gajewski and others 2009; Fæste and Plassen 2008). As previously described, immunoassay is a convenient and rapid method that can recognize a target protein (analyte) through a specific antibody-antigen interaction. Immunoassays have been reported for valuable fish species identification such as grouper (Asensio and others 2003), red snapper (Huang and others 1995), or imported fish species identification, like pangasius catfish (Hsieh and others
However, these assays focus on the identification of individual fish species and none distinguish fish from other animals. This is because the primary purpose of fish species identification is to prevent substitution of high-valued fish species with low-valued fish species for economic gain in retail chains or restaurants. A sandwich ELSIA using polyclonal antibodies has been developed for the detection of fish in food (Fæste and Plassen 2008), but unfortunately, twelve of thirty two fish species tested showed low recovery rates and could not be detected by this assay. In addition, the polyclonal antibody utilized exhibits cross-reactivity with European squid. Therefore, at present, no effective immunoassay for fish detection is available.

The main difficulty involved in the development of an immunoassay for fish detection has been the sheer diversity of food fish species and the lack of suitable marker protein that would serve as an antigen for all fish species, both raw and cooked. The fish marker protein selected must be stable as well as fish-specific for most commonly consumed food fish species because most proteins are not thermal-stable, and any food processing may also degrade or denature the protein. Although raw fish dishes such as sushi (raw fish with rice) and sashimi (raw fish only) are becoming more popular in the US, as yet there are no reports of a suitable marker protein to represent fish components in food products. Therefore, the overall goal of this study is to characterize a marker protein and then use this marker protein to develop a sandwich ELISA for fish detection to ensure the safety of food and feed products.

In order to develop a sandwich ELISA for fish detection, MAb 8F5 was previously developed in our laboratory using the protein extract from cooked red snapper as the immunogen. Our preliminary results showed that MAb 8F5 cross-reacted to all cooked extracts of 55 common food fish species with no cross-reaction with samples from land animals, shellfish or food additives (Fig.1). The antigenic protein of MAb 8F5 is a 36 kDa thermal-stable protein. Because this 36 kDa protein appears among all fish species and is thermal-stable, it has the potential to be a marker protein for the development of a sandwich ELISA. A literature review revealed that fish tropomyosin has a similar molecular weight and thermal-stability, so the first objective of this research was to verify that the 36 kDa protein is indeed fish tropomyosin and to investigate the conserved region of the 36 kDa protein using MAb 8F5. The second objective of this research was to move on to develop a sandwich ELISA using the 36 kDa protein as the target protein for the detection of fish in food products.
2. Hypotheses

Fish is prepared for consumption using a wide range of cooking styles or processing methods, including smoking, salting and canning. Heat treatment significantly affects the properties of proteins by inducing denaturation and aggregation, thus causing changes in solubility and immunoreactivity (Beran and others 2007; Cheng and Parrish 1979). Although most fish muscle proteins lose their extractability after heat treatment, several thermal-stable proteins remain soluble in the extraction buffer. The preliminary data for this study identified the 36 kDa protein as one of the major thermal-stable soluble proteins in fish protein extracts (Fig. 3). Furthermore, the 36 kDa protein is an antigenic protein of fish-specific MAb 8F5 (Fig. 2). This suggests that the fish 36 kDa protein may have a fish-specific region in its amino acid sequence.

To further investigate this fish 36 kDa protein, fish tropomyosin was selected as a candidate protein based on its molecular weight and thermal-stability. The molecular weight of tropomyosin ranges from 33-38 kDa, depending on the species. Preliminary results showed that the 36 kDa protein is capable of recognition in the great majority of the fish species tested and shows double protein bands for yellowfin tuna samples analyzed by SDS-PAGE (Fig. 2A & Fig. 3B). Fish tropomyosin, a 36 kDa protein, has two isoforms in several fish species such as tuna and puffer fish, but in most fish species has only one form of tropomyosin (Huang and Ochiai 2005; Heeley and Hong 1994). Because the characteristics of fish 36 kDa protein are similar to fish tropomyosin, we hypothesized that the 36 kDa antigenic protein of fish-specific MAb 8F5 is likely to be fish tropomyosin.

Development of a fish-specific sandwich ELISA depends on two critical elements. First, a marker protein which is found in both raw and cooked fish ingredient in food and food products must be identified. The 36 kDa protein is known to be a common protein in fish muscle among fish species and also a major thermal-stable protein in cooked fish extracts. Therefore, the fish 36 kDa protein was selected as the marker protein for fish detection. Second, a pair of antibodies specific to the fish thermal-stable marker protein is required. Although we had a fish-specific MAb 8F5, another antibody was needed to make a pair, specifically an antibody that can recognize different epitopes other than the epitope of MAb 8F5. The fish 36 kDa protein would serve as the immunogen to develop polyclonal antibodies. The advantage of using a polyclonal antibody is its ability to recognize multiple epitopes and hence reduce epitope competition. The
final objective of this research was, therefore, to develop another antibody that would be suitable for use in developing the sandwich ELISA. After optimization, this sandwich ELISA would be able to detect fish and fish products even after processing involving heat treatment.

3. Objectives

The specific objectives of this study were:

1): to characterize the 36 kDa thermal-stable antigenic fish muscle protein as a potential fish marker protein

2): to develop a sandwich ELISA using the 36 kDa protein as target protein for the detection of fish in food products.

After the extensive literature review in Chapter 1, Chapter 2 reports on the characterization of the antigenic protein of fish-specific MAb 8F5. This antigenic protein is a thermal-stable 36 kDa protein present in all the fish species tested. The characterization was performed via molecular weight migration in urea gel, the immunoreactivity to MAb 8F5, amino acid composition and protein sequence. Chapter 3 describes the development of a fish-specific sandwich ELISA using the fish 36 kDa protein as the target protein to detect the presence of fish muscle. The development of a sandwich ELISA requires a matched pair of antibodies to recognize different epitopes on the target protein, so polyclonal antibodies were produced against the fish 36 kDa protein. The resulting matched antibodies were then used as a pair in the sandwich ELISA. Finally, the species specificity, detectability of fish products, the limit of detection and reproducibility were evaluated for the optimized assay.
CHAPTER ONE

LITERATURE REVIEW

1.1 Importance of developing a fish immunoassay

1.1.1 Fish allergy and its associated regulations for consumer protection

Seafood includes vertebrate fin fish such as cod, salmon, and tuna, crustaceans such as shrimp, crab, and lobster, and mollusks such as squid, scallop, clams, and snails. Seafood consumption is increasing in the United States. According to data obtained from the National Oceanic and Atmospheric Administration (NOAA) Fisheries Service, Americans consumed 16 pounds of fish and shellfish per person in 2010, a 7.4% increase compared to 1998’s consumption of 14.9 pounds (NOAA, 2011).

Unfortunately, fish and shellfish are allergenic foods to sensitized people. The major allergic reaction to seafood is an immunoglobulin (Ig) E-mediated immunology reaction triggered by ingestion, touch (Bernhisel-Broadbent and others 1992a; James and others 1997) and/or inhalation (Jeebhay and others 2001) of allergic proteins. The common symptoms of fish and shellfish allergy include urticaria, angioedema, asthma, rhinitis, vomiting, diarrhea and anaphylaxis (David 2000; Helbling and others 1996).

Lehrer and others (2003) reviewed research published between 1966 and 2000 and found that allergic reactions have been recorded by those engaged in a wide variety of seafood-related occupations. These workers include fishermen, fish and prawn workers, seafood processing workers, canners, restaurant cooks, and a number of other workers associated with other aspects of the seafood industry and seafood products. Cartier and others (1984) reported that workers in the snow crab industry are exposed to occupational allergens through direct contact with seafood products, including the inhalation of seafood and water droplets generated during seafood processing. Over one third reported suffering from asthma, 18% reported rhinitis or conjunctivitis, and around 24% reported some sort of skin rash among the 303 crab workers surveyed (Cartier and other 1984).
Studies of the prevalence of fish allergy indicate that fish allergy is a worldwide issue for food safety. In Norway, around 0.1% of the total population suffer from a fish allergy (Aas 1987). In Italy, De Martino and others (1993) studied 558 children and deduced that 0.4% to 0.5% of the children were allergic to cod. In Spain, an estimated 30% of children have a fish allergy (Crespo and others 1995) and similar results have been reported in Finland, Sweden and Japan (Hamada and others 2001; Saarinen and Kajosaari 1980; Björnsson and others 1996). Sicherer and others (2004) were the first to report on the incidence of seafood allergy in the US, gathering data via a nationwide random telephone survey. A strong history of allergy to seafood (fish or shellfish) was reported by 2.3% of the surveyed individuals, of whom 0.4% reacted to finfish, 2% to shellfish, and 0.2% to both. Subjects were neither evaluated for IgE sensitization nor verified by means of challenge testing so these figures may be overestimates, but nonetheless this result might not be too far from the true prevalence of fish and shellfish allergy (Sicherer and others 2004).

Fish allergy has cross-reactivity between different fish species. The major fish allergen, parvalbumin, is found in cod, mackerel, herring, and plaice (Helbling and others 1999; Hansen and others 1997). Parvalbumin in fish species from both salt water and fresh water can cause allergic reactions (Swoboda and others 2002; Bugajska-Schretter and others 1998a). Bugajska-Schretter and others (1998) prepared fish extracts from cod, tuna, salmon, perch, carp, and eel to characterize the cross-reactivity of parvalbumin in these 6 fish species. The results indicated that fish allergic patients may be allergic to two, three or more species of fish. Based on these studies, it is critical for fish allergic patients to avoid all forms of fish in their foods.

To protect consumers from consuming allergic ingredients accidentally and unknowingly, the Codex General Standard for the Labeling of Prepackaged Foods, the European Union, the USA and other countries continue to develop regulations to ensure that allergenic foods and food ingredients are properly labeled. In the US, the Food Allergen Labeling and Consumer Protection Act (FALCPA) was enacted in August 2004 and took effect January 1, 2006 (Public Law 108-282). It addresses, among other issues, the labeling of foods that contain certain food allergens. The purpose of the act is to ensure that consumers can easily and accurately identify food ingredients that may contain allergen sources.
The eight “major food allergens” regulated under FALCPS are milk, eggs, fish, crustacean shellfish, peanuts, tree nuts, wheat, and soy. These foods, and any ingredients derived from them, must be declared in plain language on the ingredient list, by the word “Contains” followed by the name of the major food allergen, or by an additive statement in the list of ingredients, for example “albumin (egg)”. These major food allergens must be listed even in colors, flavors, or spice blends. In addition, manufacturers must list the specific nut or seafood that is used (for example almond, walnut, cashew; or tuna, salmon, shrimp, or lobster). In accordance with the Federal Food, Drug, and Cosmetic Act, a noncompliant company will be subject to civil and criminal penalties and food products containing undeclared allergens may be subject to recall.

1.1.2 Concerns regarding the use of fishmeal in animal feed

Fishmeal is a common ingredient, providing dietary protein in animal feed. Fishmeal is produced by cooking, pressing and grinding both whole fish and offal from fish. Bovine spongiform encephalopathy (BSE) is a transmissible spongiform encephalopathy (TSE), a fatal brain disease. Fishmeal itself entails no risk of TSE, but it is banned for use in ruminant feed because it could be accidentally contaminated by banned meat tissue during processing, packaging and storage. In the European Union, a temporary ban was placed on the use of fishmeal in ruminant feed in 2001 (EC 2000/766 and 2001/9). It was then officially banned for ruminant feedstuff and included in the 2003 TSE regulations (EC 999/2001). Japan and Australia have also banned the use of fishmeal to feed ruminants, in 2001 and 2002, respectively. In the European Union, microscopy is the only officially approved method for identifying processed animal protein (including fishmeal) in animal feed. Microscopy identifies animal species by the characteristics of bones, feathers or other fragments found in animal feeds. Generally, microscopy is a time-consuming method that requires expensive instruments and well trained analysts. A more reliable and simpler method for fish detection in food and feed is therefore urgently needed in order to protect fish allergic patients and reduce the TSE risk to human and animal health.
1.2 Currently available methods for the detection of fish in food

The major purpose of detection is to identify the presence of allergenic food ingredients. Currently, several methods have been developed for the detection of fish in food. These methods target a specific protein or a particular DNA fragment as a marker for fish detection. Microscopy is the officially sanctioned method (Commission Directive 2003/126/EC) in the European Union for detecting and identifying animal constituents, but this method is expensive, time consuming and relies on well-trained specialists.

DNA-based methods are based on the amplification of specific DNA fragments in a polymerase chain reaction (PCR) using a primer that is keyed to a specific DNA region found only in the offending food. The advantages of DNA-based methods are that they are highly specific and have detection limits as low as 10 mg/kg for almonds, hazelnuts, soy, milk and peanuts (Poms and others 2004). The quantity of the allergenic proteins present can also be estimated by real-time PCR via comparison to a standard curve. Compared to proteins, DNA has a relatively stable level between individual organisms or in different growth stages. However, the major limitation of DNA-based methods is that they are an indirect method for target protein detection and the quantity of target DNA fragments does not directly represent the amount of target protein in the sample tested. Although DNA is considered more thermal-stable than protein, DNA cannot withstand severe food processing such as deep frying (Hsieh and others 2004). DNA-based methods also require expensive equipment and well-trained person. These limitations mean that DNA-based methods are not user-friendly.

Currently, the DNA-based methods that have been developed for fish detection include PCR-sequencing, PCR-restriction fragment length polymorphism (PCR-RFLP), and real-time PCR, but all these focus primarily on the identification of fish species because they were originally developed to protect consumers against the substitution of low value fish for high value fish. They are therefore able to identify high value fish species such as mackerel, cod, herring, salmon and tuna (Bartlett and Davidson 1992; Rehbein and others 1999). The only DNA-based method for general fish detection is a commercial kit, SureFood Allergens Fish real-time PCR-test kit, developed and manufactured by Congen Biotechnologie GmbH and
distributed by R-Biopharm (Marshall, MI, USA). However, there is no any further information on the target DNA or any independent reports evaluating its performance.

The major protein-based methods for fish detection include isoelectric focusing (IEF) and immunoassay. IEF can identify fish species by the specific pattern of fish proteins in IEF gel. The proteins in fish extract are separated based on their isoelectric points, producing a characteristic pattern. IEF is the official method for fish species identification in the United States. The IEF profiles of 94 fish species are available in a regulatory fish encyclopedia (REF) issued by the U.S. Food and Drug Administration (FDA 2012). This technology is useful for identifying fish species such as red snapper, European perch, Nile perch, European pikeperch and Sunshine bass (Hsieh 1997; Berrini and others 2005). However, it is not suitable for samples containing more than one fish species or other meats. In addition, it is impossible to establish a complete IEF data base for all food-fish.

Immunoassay, a protein-based method, detects target food proteins through specific antibody-antigen interactions. The target proteins can be food allergenic proteins or marker proteins that indicate the presence of the allergenic foods. The antibody for detection may be collected from sera from patients with an allergy or from immunized animals. However, using human IgE to develop an immunoassay is difficult for standardization and commercialization, so most of the antibodies used are raised against target proteins in animals. Immunoassays, including ELISA and lateral-flow device methods, are becoming very popular and are now routinely using in industrial settings and governmental agencies. A wide range of immunoassays have been developed and become commercial products for the detection of food allergens. The lateral-flow device method is especially user-friendly, requiring no special equipment or training (Posthuma-Trumpie and others 2009).

One important requirement of immunoassays for food detection is to be able to select and identify a suitable marker protein. The development of a reliable method for fish detection in food and food products requires the selection of an appropriate marker protein that will unambiguously indicate the presence of fish ingredients in food and food products. The allergenic protein in fish is obviously the first choice, but as with other allergenic foods, fish contains more than one allergenic protein. Currently, the fish allergenic proteins that have been
identified include parvalbumin (Elsayed and Aas 1971), collagen (Hamada and others 2001) and aldehyde phosphate dehydrogenase (APDH) (Das Dores and others 2002). These fish allergenic proteins were identified using sera from patients with fish allergies raised against fish extracts. The clinical sensitivity of these allergenic proteins for fish allergic patients is still not fully understood and often varies considerably between individual patients. Taylor and others (2002) reviewed studies of fish allergy conducted by several groups and found that the threshold doses of fish for fish allergic patients was reported to be anywhere between 5 mg (cod) and 6000 mg (plaice). The threshold level of fish in food has not been determined for the purposes of legal labeling requirements. Also, the quantity of the allergenic proteins may be very low in fish muscle. Using a less abundant protein as the target protein will inevitably decrease the sensitivity of the immunoassay. Furthermore, food processing such as heat treatment may denature or change the allergenic protein structure and thus increase the difficulty of detection. Bernhisel-Broadbent and others (1992b) indicated that boiled fish samples showed a strong decrease in reactivity with IgE.

The alternative is to select a suitable marker protein which is abundant in fish muscle and resistant to food processing. Heating is the most common method for fish preparation for consumption. However, most fish muscle proteins are heat labile so the epitopes on the target native protein may not be recognized by the antibody after cooking. However, this problem is often encountered with an immunoassay and can be overcome by using an antibody developed against thermal-stable proteins. Examples include porcine troponin I (Chen and Hsieh 2002; Hsieh and Bridgman 2004) for bovine, ovine and porcine detection; bovine h-caldesmon for bovine detection; (Kim and others 2006) and fish parvalbumin for fish allergen detection (Gajewski and Hsieh 2009) in both raw and cooked food products. Another important requirement is the selectivity and cross-reactivity of the antibody in the immunoassay. The antibody for fish detection must have cross-reactivity to all fish species, while the marker protein must contain fish-specific regions in order to distinguish the fish from other animal or plant ingredients.

Currently, the available immunoassays for fish detection are all designed for fish species identification including grouper, wreck fish, Nile perch (Asensio and others 2003), red snapper (Huang and others 1995) and pangasius catfish (Hsieh and others 2009). These immunoassays
were developed to discourage fraudulent fish substitutions in restaurants or retail markets. None of these immunoassays are capable of cross-reacting with all fish species. Furthermore, there are no protein-based commercial products for fish detection. These suggest that it is very difficult to find a suitable marker protein for the general detection of fish, which encompasses a large number of species. This is likely to be the major reason why there is as yet no commercially available immunoassay for the detection of fish in food or feed.

1.3 Thermal-stable proteins in fish muscle

The thermal-stability of fish muscle proteins has been examined by differential scanning calorimetry (DSC), which examines protein denaturation under a linear temperature increase. The thermal-stability of several fish muscle proteins is known to be different in different fish species. The thermal-stability of myofibrillar protein among various fish species by DCS was studied by (Howell and others 1991, who analyzed fish species from tropical water including catfish, carp, Nile perch, red snapper, red mullet, and sea bream, as well as cold-water fish species including trout and cod. Their results showed that the temperature of myofibrillar protein denaturation in catfish, a tropical species (43.5°C) is 11°C higher than that in a cold water species, cod (32.6°C). This suggests that the habitat temperature of different fish species may affect the thermal-stability of their fish muscle proteins.

Myosin is the most abundant myofibrillar protein in muscle. The molecular weight of fish myosin is around 500 kDa and it contains 2 heavy chains and 4 light chains. The denaturation temperature of fish myosin undergoing DSC analysis was reported to be 40–50°C (Davies and others 1988). The second most abundant myofibrillar protein is actin (45 kDa). Carp actin is known to lose the ability to polymerize between 45°C and 55°C (Ooi and others 2008) and its temperature of denaturation is 75°C (Akahane and others 1985). Fish tropomyosin is a 36 kDa regulatory protein involved in muscle contraction. Huang and Ochiai (2005) isolated the tropomyosin of six fish species from different habitat temperatures and evaluated the thermal-stability by DSC. They found that all the fish tropomyosin tested showed species-specific thermal-stability, with denaturation temperatures ranging from 37.7°C to 46.5°C. In addition, all
the fish tropomyosin tested showed the ability to refold after denaturation, which suggests fish tropomyosin may have a high potential for heat resistance.

Allergenic proteins are usually thermal-stable because they continue to cause allergic reactions after heat treatment. There is a variety of proteins in fish muscle tissue, only a few of which are known allergens. Currently identified fish allergenic proteins include parvalbumin (Elsayed and Aas 1971), collagen (Hamada and others 2001b) and aldehyde phosphate dehydrogenase (APDH) (Das Dores and others 20002).

The first major allergen in fish to be identified was parvalbumin, Gad c1, which is a 12 kDa protein from codfish (Elsayed and Aas 1971). Parvalbumin is a calcium-binding protein in muscle tissue and plays an important role in muscle physiology. The allergic cross-reactivity of parvalbumin has been reported in many other fish species including salmon, carp, catfish, dogfish, snapper and mackerel (Lindstrom and others 1996; Hamada and others 2003b; Wopfner and others 2007; James and others 1997). Fish parvalbumin is also a thermal-stable allergen. The parvalbumin in crude protein extracts of cooked (100°C for 10min) fish still could be recognized by IgE from fish allergic patients (Kobayashi and others 2006). However, the allergenic activity of cooked fish parvalbumin was weaker than in uncooked fish parvalbumin (Bernhisel-Broadbent and others 1992b).

The second major fish allergenic protein is collagen. Hamada and others (2001) identified two allergic proteins with high molecular weights of 120 and 240 kDa. The allergenicity of these proteins was confirmed in a competitive ELISA using sera from fish allergic patients. The 120 kDa protein has been identified as a α-chain of collagen and the 240 kDa protein as a β-chain of collagen, a dimer of the α-chain. Amino acid analysis revealed that these proteins contain two collagen-specific amino acids, namely hydroxyproline and hydroxylysine, thus further confirming that the allergenic protein is indeed collagen. Hamada and others (2003a) reported that purified fish collagen from Japanese eel, alfonsin, mackerel, skipjack and bigeye tuna were recognized by IgE from fish allergic patients. This result suggests that fish collagen has cross-reactivity among different fish species.
The third fish allergenic protein is aldehyde phosphate dehydrogenase (APDH), a protein first reported in cod (Das Dores and others 2002). The cod APDH is a 41 kDa protein identified by IgE from fish allergic patients by Western blot (Das Dores and others 2002).

Yamada and others (1999) showed that sera samples from 8 fish allergic patients showed anaphylaxis to both raw and cooked fish extracts from two different kinds of tuna (albacore tuna and yellowfin tuna). Helbling and others (1992) also reported that fish allergic patients reacted to a cooked fish product, in this case Alaskan pollock derived surimi. Rosmilah and others (2005) found several fish allergens with molecular weights of 151, 125 and 90 kD in cooked extracts of red snapper and golden snapper. These results suggest that there are several thermal-stable proteins in fish that are allergens.

1.4 The characteristics of tropomyosin

The antigenic protein MAb 8F5 is a thermal-stable, 36 kDa soluble protein. Based on its molecular weight and thermal-stability, tropomyosin was selected as a possible candidate for the antigenic protein MAb 8F5 in this study. Consequently, the following sections focus on the physiochemical properties of tropomyosin.

Tropomyosin was first isolated from rabbit skeletal muscle (Bailey 1946). Hamoir (1951) was the first to isolate fish tropomyosin from carp muscle, using Bailey’s method. The molecular weight of fish tropomyosin was identified as 36 kDa. Since then, tropomyosin has been isolated from many different fish species, including Atlantic salmon (*Salmo salar*), rainbow trout (*Salmo gairdneri*), bluefin tuna (*Thunnus thynnus*), pufferfish, (*Takifugu rubripes*), walleye pollock (*Theragra chalcogramma*), white croaker (*Pennahia argentata*), largemouth bass (*Micropterus salmoides*), cod (*Gadus morhua*) and zebrafish (*Danio reio*) (Odense and others 1969; Heeley and others 1995; Huang and Ochiai 2005; Thys and others 2001; Georgijevic and others 2007). The molecular weight of isolated fish tropomyosin showed slight differences among fish species. Heeley and Hong (1994) isolated fish tropomyosin from 17 fish species and reported that the molecular weights of salmon, mackerel, char, herring, thorny skate and lamprey were higher than the other species tested. The theoretical molecular weight of fish fast skeletal
muscle α-tropomyosin is around 33 kDa, as determined by MALDITOF. All the fish molecular masses from MALDITOF are slightly larger than this calculated value based on their amino acid sequences (Huang and Ochiai 2005; Huang and others 2004). The molecular weight of fish tropomyosin appears slightly higher than 33 kDa at around 36 kDa in SDS-PAGE. The phosphorylation and oxidation of fish tropomyosin affects the mobility of fish tropomyosin in SDS-PAGE (Heeley and Hong 1994). The isoelectric point (pI) value of *Drosophila* tropomyosin reported by Bautch and Storti (1983) was 5.0. The pI value of mammalian tropomyosin from rabbit has been measured at from 5.1 to 5.0, depending on the different isoforms (Bronson and Schachat 1982). The isoelectric point of fish tropomyosin in bluefin tuna is reported to be slightly lower, at 4.5 (Huang and others 2004).

### 1.4.1 Fish tropomyosin isoforms and distribution

Tropomyosin in mammalian skeletal muscle consists of two types, α and β (Cummins and Perry 1972, 1973). Both the α- and β- tropomyosin have highly conserved amino acid sequences (Stone and Smillie 1978; Mak and others 1980). Dhoot and Perry (1979) suggested that the α tropomyosin is specific to fast-twitch muscle and β tropomyosin is specific to slow-twitch muscle. Fish tropomyosin in skeletal muscle also has two isoforms, α-tropomyosin and β-tropomyosin (Huang and Ochiai 2005; Heeley and Hong 1994). However, unlike mammalian samples, only some fish species have both isoforms of fish tropomyosin. Huang and Ochiai (2005) isolated tropomyosin from six different fish species, including walleye pollock, white croaker, Atlantic salmon, puffer fish, bluefin tuna and zebrafish and reported that one-dimensional polyacrylamide gel analysis of isolated tropomyosin from these fish species revealed that some fish species, in this case mackerel, bluefin tuna and puffer fish have both α- and β-tropomyosin isoforms while other fish species, namely walleye pollock, white croaker and cod, only have α-tropomyosin in skeletal muscle (Huang and Ochiai 2005; Heeley and Hong 1994). The molecular weight of salmon tropomyosin is close to that of one of the bluefin tuna tropomyosin isoforms. In puffer fish, α-tropomyosin showed 87% identity of amino acid sequence with β-tropomyosin (Toramoto and others 2004). The differences between these isoforms of fish tropomyosin require further study.
Fish tropomyosin is widely distributed throughout all different muscle types: skeletal muscle, cardiac muscle and smooth muscle. The tropomyosin isolated from salmonid fish such as Atlantic salmon (Salmo salar) and rainbow trout (Salmon gairdneri) has been found in various muscle tissues including fast muscle, slow muscle, cardiac muscle, stomach, tongue and cheek (Heeley and others 1995). The quantity of fish tropomyosin expressed in different muscles is consistent from head to caudal fin. Thys and others (2001) investigated the muscle protein component of largemouth bass (Micropterus salmoides) using SDS-PAGE and found no variation in the tropomyosin concentration in different longitudinal muscle positions. These published data indicate that fish tropomyosin is present in all types of fish muscle and has an even distribution throughout the skeletal muscles.

1.4.2 The allergenicity of tropomyosin

Tropomyosin is also reported to be a major allergen in various invertebrates such as crustaceans (shrimps, crabs, and lobsters), mollusks (squid, snails and oysters), arachnids (dust mites), and insects (cockroaches, midges) (Shanti 1993; Chu 2000; Ishikawa and others 2001; Miyazawa and others 1996; Martínez and others 1997). However, unlike invertebrate tropomyosin, the allergenicity of vertebrate tropomyosin has not been reported in humans. Mikita and Padlan (2007) suggested that the difference in allergenic reactions between the invertebrate and vertebrate forms may be a result of pepsin A digestion. Pepsin A is a major gastric protease and prefers peptide bonds following phenylalanine and tyrosine. The major products of pepsin A are large fragments and some free amino acids. Comparing the amino acid sequences of tropomyosin from invertebrates (crabs, shrimps, cockroaches and dust mites) and vertebrates (chicken, cattle, rabbits and salmon), invertebrate species have larger pepsin A fragments than vertebrate species. The vertebrates have 102 residues (36%) of 284 total residues in the longest digested fragment, while crabs, shrimps and cockroaches have 153 residues (54%) and dust mites have 162 residues. The higher chance and severity of allergic reaction to tropomyosin from shrimps, crabs, cockroaches and dust mites may be a result of these longer digested fragments of tropomyosin entering the blood stream (Mikita and Padlan 2007).
1.4.3 Thermal-stability of tropomyosin

Tropomyosin has been reported to be a thermal-stable protein in both invertebrate and vertebrate species. Ishikawa and others (2001) heated crude extracts in a water bath for 10 min to isolate tropomyosin from octopus muscle. The isolated octopus tropomyosin was not denatured and retained its allergenicity. Odense and others (1969) isolated fish tropomyosin from cod skeletal muscle after heat treatment and compared it with tropomyosin isolated from raw samples. The heat-treated samples of minced cod were boiled for 10 min and the tropomyosin isolated by salt precipitation. The subsequent gel electrophoresis patterns of the unheated and heated tropomyosin samples were similar and the amino acid components had only a few differences. The crystallized structures of the fish tropomyosin were also very close to each other under photomicrography (Odense and others 1969). Tropomyosin is a coiled-coil protein with heptad repeats \(abcdefg\) throughout the entire length of the molecule. In heptad repeats, the \(a\) and \(d\) positions form the helix–helix interface, while positions \(e\) and \(g\) form the salt-bridges and the remaining positions (\(b, c,\) and \(f\)) are exposed to the surface of the molecule (Woods 1976). Compared to the different fish \(\alpha\)-tropomyosin amino sequences, the 27th, 83th, and 135th residues from the N-terminus vary between fish species. These residues may play an important role in the thermal stability of fish tropomyosin (Huang and Ochiai 2005).

The thermal-stability of tropomyosin between different fish species has also been investigated using circular dichroism (CD) and DSC to monitor the effects of heat on protein denaturation (Huang and Ochiai 2005; Ochiai and others 2003; Huang and others 2004). The CD spectrometer measures conformational changes in the protein under different temperature environments. All the fish tropomyosin tested contained high \(\alpha\)-helix structure at 4°C but as the temperature increased the \(\alpha\)-helix structure started to unfold at 20°C and no \(\alpha\)-helix structure was observed above 50°C. In the case of walleye pollock, the \(\alpha\)-helix structure content of tropomyosin was reported to be 95.5% at 4°C and 89% at 20°C. After heating and cooling, the \(\alpha\)-helix structure content was reduced to 82.3% at 4°C (Ochiai and others 2003). The DSC results indicated that the range of melting temperature (Tm) values of tropomyosin ranged from 37°C to 44°C for different fish species (Huang and Ochiai 2005). The major endothermic peaks appeared at around 40°C for all the tropomyosin samples examined. However, bluefin tuna and puffer fish were both found to contain two tropomyosin isoforms with two different melting temperatures.
The Tm values of the β-tropomyosin isoform obtained from bluefin tuna and puffer fish were 29°C and 26°C, respectively, much lower than those of their α-tropomyosin isoforms. The variation in the thermal stability of tropomyosin among fish species may be caused by a difference in their hydrophobic interfaces. Greenfield and Hitchcock-DeGregori (1995) reported that the hydrophobic interface of a coiled coil is important for stabilizing tropomyosin molecules. The amino acid sequences within the hydrophobic core of tropomyosin in different fish species are very similar. Furthermore, these differences in the Tm values among fish species are likely to be related to their temperature of habitation and swimming type (Huang and others 2004).

1.4.4 The molecular weight of tropomyosin

Tropomyosin was first isolated by Bailey (1946) from rabbit muscle tissue and the molecular weight of monomer rabbit tropomyosin is around 33 kDa (Smillie 1979). Native tropomyosin is a dimer with a molecular weight of 68 kDa (Woods 1967). In recent research, Lassing and others (2010) further characterized rabbit tropomyosin under physiological salt conditions. Here, the purified rabbit tropomyosin appeared as a single protein with a molecular weight of 130 kDa, four times the molecular weight of rabbit tropomyosin. It has been suggested that native tropomyosin is a tetramer in physiological salt solution. After dissociation, rabbit tropomyosin can separated into two major isoforms with similar amino acid composition and molecular weight (Cummins and Perry 1973). The major difference between these two isoforms is their cysteine content: the α-form contains one cysteine residue and the β-form contains two cysteine residues. There are no significant biological differences between these two isoforms (Cummins and Perry 1974). These studies indicate that native rabbit tropomyosin is a tetramer that contains four heterogeneous units, each with a molecular weight of around 33kDa.

Tropomyosin exhibits different behaviors under SDS electrophoresis depending on the absence or presence of urea. In the absence of urea, the molecular weight of rabbit tropomyosin is around 33 kDa (Bailey 1946), but Sender (1971) reported that the molecular weight of rabbit tropomyosin migrates from 33 kDa to around 50 KDa in the presence of urea. None of the other muscle fibril proteins’ molecular weights appeared to be affected by urea (Sender 1971). Shanti (1993) extracted tropomyosin and allergenic protein (Sa-II) from shrimp. In the presence of urea,
the molecular weight of the shrimp tropomyosin and Sa-II both shifted to 50 kDa. Vejborg and others (2008) reported a similar observation for cod tropomyosin. The reason for this molecular weight migration is that the SDS binding amount of protein is reduced in the presence of urea (Takagi and Kubo 1979). These results indicate that the α-helical protein reduces the 50-70% SDS binding amount. Therefore, the conformation of the SDS-protein complex is changed by adding urea and the effective size of the SDS-protein becomes greater in the presence of 8M urea than in the presence of SDS alone. Increasing the size of the SDS-protein complex would thus contribute to the reduction in protein mobility.

1.4.5 The structure of tropomyosin

Tropomyosin is a two-stranded muscle protein that has long been considered the archetype of α-helical coiled coils (Fig. 6) (Whitby and Phillips Jr 2000). The protein sequence of tropomyosin consists of a short-range seven-residue (‘‘heptad’’) repeat of the form \((a-b-c-d-e-f-g)\), where \(a\) and \(d\) are usually apolar residues. The \(a\) and \(d\) residues form a left handed apolar stripe along the surface of the right-handed α-helices. Two major functions of tropomyosin are cooperative binding to F-actin and cooperative inhibition or activation of actomyosin Mg-ATPase (Hammell and Hitchcock-DeGregori 1996). The N- and C-termini of the tropomyosin molecule overlap to form a fibrous polymer (Greenfield and others 1998). The tropomyosin lies along the grooves of the F-actin at the ratio of one molecule to seven actin molecules (Muthuchamy and others 1997; Borovikov 1999). The interlocking of these residues is a ‘‘knobs-into-holes’’ pattern, where connections are made by winding two (or three) helices around one another to produce a coiled-coil molecule (Crick 1953). This structure provides good stability for highly charged polypeptide chains in an aqueous environment (Cohen and Parry 1990). McLachlan and others (1975) suggested that tropomyosin is the first α-helical coiled coil protein and most isoforms (yeast excepted) have been shown to consist of 40 continuous heptads, which is unusual in fibrous proteins. Tropomyosin molecules bond head to tail, with a short overlap of about nine residues, to form an essentially unbroken coiled-coil cable that winds around the actin helix. In vertebrate striated muscle, the contractile activity of myosin is controlled by a calcium sensitive complex of troponin and tropomyosin. Salt links between the residues \(e\) and \(g\) on neighboring chains may also provide an additional stabilization of the coiled coil.
1.5 Potential fish-specific regions of the fish tropomyosin amino acid sequence

Tropomyosin has a high identity of amino acid sequence among different fish species. In a study by Ochiai and others (2003), the tropomyosin cDNA from walleye pollock (Theragra chalcogramma) fast skeletal muscle was cloned and its full-length DNA found to contain 1168bp, with a 855 bp open reading frame encoding 284 amino acids. Fish tropomyosin may be expressed as α-tropomyosin only or as both α- and β-tropomyosin, depending on the fish species. To further compare the differences between fish α- and β- tropomyosin, Toramoto and others (2004) cloned both α- and β- tropomyosin from pufferfish (T. rubripes) by cDNA expression. The pufferfish α- and β-tropomyosin differed by 37 amino acid residues in a total of 284 residues. The sequence identity was 87.0%. In a comparison of walleye pollock tropomyosin with α-tropomyosin from white croaker, Atlantic salmon, pufferfish, bluefin tuna and zebrafish the amino acid identity ranged from 94.7 to 97.2% (Huang and Ochiai 2005). Due to the high identity among different fish species, it would thus appear to be feasible to identify a conserved region in the tropomyosin of all fish species that is not shared by any of the non-fish species for antibody binding.

Huang and others (2004) cloned tropomyosin cDNA from dorsal fast skeletal muscle in bluefin tuna. Aligning the α-tropomyosin amino acid sequences of bluefin tuna with various animals including fish species, the amino acid sequence identity was in the range of 93.7–98.6%. According to their amino acid identity, the difference between fish species was lower than between land animals and fish species.

1.6 The effect of heat treatment on the epitope.

For the detection of allergenic proteins, antibodies (IgG) have been generated in immunized animals (mice, rats, rabbit, goat or other animals). These antibodies can be applied in immunoassays to detect allergenic proteins or serve as suitable marker proteins to indicate the presence of food allergens in food and food products.
The operating principle of an immunoassay is based on an antibody-antigen interaction in which an antibody recognizes specific regions (epitopes) on the target protein. The epitopes on an antigenic protein are either linear or conformational, depending on its structure. A linear epitope is a continuous region on the amino acid sequence of the target protein, while a conformational epitope is a 3-dimensional structure which is made up of discontinuous segments on the amino acid sequence. Epitopes may have both linear and conformational characteristics.

A number of recent studies have focused on IgE-binding epitopes on allergenic proteins. Their findings indicate that the immunoreactivity of epitopes can be affected by both physical and chemical treatments. For example, the epitope may be liable to chemical treatment such as proteolysis or a severe pH environment: allergenic proteins, bet v1, with conformational IgE-binding epitopes lead to oral syndromes, but their allergenicity is lost after digestion (Schimek and others 2005). Furthermore, heat treatment is a common method for reducing the allergenicity of food allergens (Chen and others 2006). These studies of IgE-binding epitopes indicate that the stability of the epitopes play an important role in the binding ability of the antibodies.

Any food processing such as boiling, frying, grilling, drying or freezing may degrade the protein or change its structure. Therefore, the thermal stability of the epitopes is critically important for immunoassays. In this study, the epitopes of fish-specific MAb 8F5 retained their immunoreactivity after heat treatment (100°C for 15 min). This observation suggests that these fish-specific epitopes are indeed thermal-stable and should therefore be good candidates for this investigation of the thermal-stable fish-specific portions of the amino acid sequence of the antigenic protein.
CHAPTER TWO

CHARACTERIZATION OF A 36 KDA THERMAL-STABLE ANTIGENIC FISH MUSCLE PROTEIN AS A POTENTIAL FISH MARKER PROTEIN

2.1 Introduction

Fish is a common allergenic food and is listed as one of the major eight allergen foods under the Food Allergen Labeling and Consumer Protection Act. Sicherer and others (2004) reported that 0.4% of the U.S. population (1.86 million people) is allergic to fish. The symptoms of fish allergy are potentially life-threatening and include angioedema, asthma, urticaria and anaphylaxis. Because there is no specific treatment to cure food allergy, the best way to prevent an allergic attack is avoidance of the allergenic food. However, simple and reliable assays for the detection of fish in food are not available. A reliable method for fish detection is therefore needed for sensitized people to minimize this risk. Immunoassay is a protein-based method that recognizes a target protein (analyte) through the reaction between an antibody and its antigen. Immunoassay has been widely used as a convenient analytical tool in industrial settings and governmental agencies for the detection of food allergens. In order to develop a reliable immunoassay for fish detection, it is important to first identify a suitable protein marker that is present in both raw and cooked fish tissue.

Monoclonal antibody (MAb) 8F5 was previously developed (unpublished data) in our laboratory by using crude protein extract of cooked red snapper (*Lutjanus campechanus*) as the immunogen. MAb 8F5 reacted with protein extracts of 55 common fish species tested without any cross-reaction with shell-fish, land animal tissue or food additives. The antigenic protein of this fish-specific antibody is a 36 kDa thermal-stable protein in the extract of cooked (100°C, 15 min) fish muscle. This 36 kDa antigenic protein is a single band separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in most of the fish species tested. Unlike other fish species, the 36 kDa protein showed double bands in tuna and had a higher molecular weight in salmon than in other fish species.
Since the 36 kDa antigenic protein is a thermal-stable and common protein among fish species, we conducted an extensive literature review of thermal-stable fish muscle proteins. Several thermal-stable proteins have been found in fish muscle, including tropomyosin (Odense and others 1969b), parvalbumin (Elsayed and Aas 1971), collagen (Hamada and others 2001a) and aldehyde phosphate dehydrogenase (APDH) (Das Dores and others 2002). Among these proteins, fish tropomyosin is the only thermal-stable protein with a similar molecular weight to the 36 kDa protein. Fish tropomyosin is a 36 kDa ubiquitous protein and has uniform distribution among fish muscles (Perry 2001; Thys et al. 2001). Huang and Ochiai (2005) have reported the double bands of tuna tropomyosin and the high molecular weight of salmon tropomyosin.

In this study we hypothesized that the antigenic protein of fish-specific MAb 8F5 is in fact fish tropomyosin and that this thermal-stable and ubiquitous muscle protein would be a strong candidate for a fish-specific marker protein for the detection of fish muscle in food and food products. Therefore, the objective of the research reported in this chapter was to characterize the 36 kDa thermal-stable antigenic fish muscle protein and assess its suitability as a potential fish marker protein.

### 2.2 Materials and Methods

#### 2.2.1 Materials

Tris-buffered saline, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl (pH 8.8), N,N,N’,N’-tetra-methyl ethylenediamine (TEMED), Precision Plus Protein Kaleidoscope Standards, 30% acrylamide/bis solution, Tris/glycine buffer, 10 × Tris/glycine/SDS buffer, supported nitrocellulose membrane (0.2 μm), and thick blot paper were obtained from Bio-Rad Laboratories Inc. (Hercules, Calif., U.S.A.). Hydrogen peroxide, horseradish peroxidase conjugated goat anti-mouse IgG (Fc specific), ABTS (2,2-azino-bis 3-ethylbenzthiazoline- 6-sulfonic acid), and β-mercaptoethanol were purchased from Sigma-Aldrich Co. (St. Louis, Mo., U.S.A.). Brom phenol blue sodium salt was purchased from Allied Chemical Corp. (N.Y., U.S.A.). Protein Assay Kit II was purchased from Bio-Rad. Sodium chloride (NaCl), sodium phosphate dibasic anhydrous (Na₂HPO₄), sodium phosphate monobasic anhydrous (NaH₂PO₄),
bovine serum albumin (BSA), sodium bicarbonate (NaHCO₃), sodium carbonate (Na₂CO₃), citric acid monohydrate, sodium dodecyl sulfate, Tween 20, and all other chemicals, reagents, syringe filters (0.45 μm Millipore, Millex R-HV), Whatman No.4 filter paper, and 96-well polystyrene microplates (Costar 9018) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). All solutions were prepared using distilled deionized water (DDI water) from a NANO pure Diamond ultrapure water system (Barnstead Intl., Dubuque, Iowa, U.S.A.). All chemicals and reagents were analytical grade.

2.2.2 Methods

2.2.2.1 Fish sample extraction

The physiological characteristics of fish muscle protein are known to be affected by the species and habitat of fish (Huang and Ochiai 2005). To control for (or minimize the effect of) these factors, common food fish species from fresh water and salt water were selected as the samples. Fresh fish muscle was minced and placed in a beaker then heated in a water bath (100°C) for 15 min. Cooked fish samples were homogenized 1 min at 13,000 rpm using the ULTRA-TURRAX T25 basic homogenizer (IKA Works, Inc., Wilmington, NC) with 5 vol. (v/w) 0.15M NaCl solution and then were centrifuged (10,000xg at 4°C, 30min) and filtered (Whatman No.4). The sample preparation of the raw fish extracts was same as for the cooked extracts, without the heating step. The protein concentrations of these protein extracts were determined by Protein Assay Kit II (Bio-Rad) and stored at -20°C until use.

2.2.2.2 Purification of fish 36 kDa protein

Fresh Atlantic salmon muscle was cut into small pieces. The fish pieces were heated in a water bath (100°C) for 15 min. Cooked fish samples were homogenized with 5 vol. (v/w) 0.15M NaCl solution and then centrifuged (5000xg at 4°C, 30min), filtered (Whatman No.4), and the protein extracts analyzed to determine their protein concentrations. The cooked salmon extracts were subjected to ion exchange chromatography using a Q-column (Hayley and others 2008). The collected fractions were analyzed by SDS-PAGE and immunoblot using MAB 8F5 to
confirm the purity. The fractions with purified 36 kDa protein were pooled. The purified salmon 36 kDa protein was stored at -80°C for further analysis.

2.2.2.3 Purification of fish tropomyosin

The isolation of fish tropomyosin generally followed the published method (Huang and Ochiai 2005). Fresh raw muscle from Atlantic salmon was homogenized with 10 vol. 0.05M KCl containing 0.002M NaHCO₃ and washed with the same solution three times. The mixtures were centrifuged at 3000Xg for 5 min. The precipitates were treated with absolute ethanol three times, and dried overnight. The completely dried powder was extracted with 10 vol. (v/w) of 20 mM Tris–HCl (pH 7.5) containing 1 M KCl and 10 mM 2-mercaptoethanol overnight. The mixture was centrifuged at 20,000Xg for 20 min. The clear supernatant was subjected to isoelectric precipitation at pH 4.5 with 1 N HCl. After centrifugation at 20,000Xg for 20 min the pellet was brought to pH 7.6 with 1 N NaOH, and subjected to ammonium sulfate fractionation to obtain a 50–60% saturated fraction. These procedures were repeated twice, and the final fraction was dialyzed against 1 mM NaHCO₃. The isolated tropomyosin was further purified by ion exchange chromatography using a Q-column (Hayley and others 2008). The fractions with tropomyosin alone were collected and stored at -80°C for further analysis.

2.2.2.4 Amino acid analysis

The purified fish tropomyosin and 36 kDa protein were hydrolyzed in 6 M hydrochloric acid (24 hours, 110°C). After hydrolysis, the sample was subjected to cation exchange chromatography and postcolumn derivation with o-phthaldialdehyde (Barkholt and Jensen 1989). The amino acid composition of the samples was analyzed by Hewlett Packard Amino Quant II system. The amino acid analysis was performed by the Protein Chemistry Laboratory at Texas A&M University.
2.2.2.5 Trypsin digestion and protein sequence analysis

Proteolytic digestion of the 36 kDa protein and the purified fish tropomyosin was performed using trypsin in 50 mM NH$_4$HCO$_3$ buffer (pH 8.5) for 1 h at 37°C in a 1:1 volume of extract:enzyme buffer. The digested fish tropomyosin and the 36 kDa protein were applied to 15% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. To visualize the protein bands, the membrane was stained with Coomassie Blue. The selected protein bands were further analyzed by a Perkin Elmer Applied Biosystems Model 494 Procise protein/peptide sequencer. The protein sequence analysis was performed by the Analytical Lab and Research Facility at Florida State University. The results of the sequencing were analyzed by Swiss Prot (Bairoch and others 2005).

2.2.2.6 Gel electrophoresis and Immunoblot

SDS-PAGE was performed according to a published procedure (Laemmli 1970) using 4% SDS- polyacrylamide stoking gel with 12% SDS- polyacrylamide separating gel. The fish extracts were loaded onto the gel in fixed volume (10μl) or fixed amounts (2µg) in each lane. Gels were run at 100V at room temperature for 120 min using Mini-PROTEAN II electrophoresis (Bio-Rad). Gels were stained with EZ Blue™ Gel staining (Sigma) according to the manufacture’s procedure. For the SDS-PAGE with urea, both the stoking gel and the separating gel contained 6M urea. Other steps were same as described above.

For the immunoblot following the SDS-PAGE, separated proteins were transferred from the SDS-gel to a nitrocellulose membrane with a 0.2 µm pore size (Bio-Rad) at 300 mA for 1 hour using Mini Trans-Blot (Bio-Rad) with transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3 (Bio-Rad). The membrane was blocked with 1% BSA in Tris buffered saline (TBS) containing 20 mM Tris, 500 mM NaCl, pH 7.4 for 1 hour. After washing in TBS with 0.05% Tween-20 (TBST), the membrane was blotted by MAb 8F5. The membrane was incubated with MAb 8F5 supernatant diluted 1:3 for 1 hour at room temperature. The excess antibody was washed away by TBST and the membranes were incubated with goat anti-mouse IgG-alkaline phosphatase conjugated antibody diluted 1:3000 for 1 hour at room temperature. The blot was visualized by incubating the membrane with 5-bromo-4-chloro-3-indolyl
phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) in 0.1 M Tris buffer at pH 9.5 for 3 min. Color development was halted by washing the membrane with distilled water. The antigenic proteins were appeared as dark purple bands. Prestained broad range protein standards (Precision Plus Protein Kaleidoscope Standards, 161-0375) were used as the molecular weight standard.

2.2.2.7 Peptide array for epitope identification

This research is based on the hypothesis that the 36 kDa antigenic protein is fish tropomyosin, which is made up of 284 amino acids (Heeley and Hong 1994). To investigate the epitope of fish-specific MAb 8F5, the amino acid sequence of Atlantic salmon tropomyosin (Swiss-Prot # Q91472) was used as template in a peptide array analysis. The customized peptide array membrane was purchased from Sigma Aldrich, USA. Each peptide was ten amino acids long with a three amino acid offset and was coated on the nitrocellulose membrane as one spot. The membrane was washed by methanol for 5 min to avoid any precipitation of hydrophobic peptides later in the washing procedure. After washing three times with TBS for 10 min at room temperature, the membrane was blocked by TBS with 3% BSA overnight at 4 °C. After blocking, MAb 8F5 (0.6 mg/ml) was diluted into 1:5,000 and was then applied to the membrane for 2 hours at room temperature with shaking. After washing with TBST three times for 10 min, the membrane was incubated with anti-IgG-HRP conjugated diluted in 1:5,000 in blocking buffer for 2 hours at room temperature. After washing the membrane three times for 10 min, the peptide bond by MAb 8F5 was detected with chemiluminescence using Immun-Star WesternC Chemiluminescent Kit (Bio-Rad). The membrane was exposed to a film (Kodak BioMax XAR film). The film was then processed by a film processor (AFP Imaging Corporation, Elmsford, NY). Film exposure and development were both performed in a dark room in the Department of Biological Science at Florida State University.

2.2.2.8 Comparison of amino acid sequence between fish and non-fish species

The sequences for the comparison of the amino acid sequence between fish and non-fish species were obtained from the data base of Swiss-Prot (Bairoch et al. 2005). The fish species
included were Brown trout (Q91490), spotted green pufferfish (Q4T142), Atlantic bluefin tuna (Q76CT4, A4PIW2), Atlantic salmon (Q91472), Zebrafish (P13104), Japanese pufferfish (Q8JIM7, Q8JIM8), Alaska pollock (Q8AV86), golden grey mullet (P84335), cardinal tetra (A7WTZ1), black prickleback (Q5YDM9), long-jawed mudsucker (Q9DFQ5), rock stickleback (Q5YDG9, Q5YDB8), and butterfish (Q5YDT5, Q5YDT1, Q5YDR3). The shellfish species included were sand shrimp (Q25456), Chinese spiny lobster (O61379), Japanese scallop (O02389, Q9NDL0, Q9GUW8), abalone (Q9GZ71), California red abalone (Q25145), Synechococcus sp (Q44119), noble scallop (Q9GZ69), blue mussel (Q25457), Mediterranean mussel (P91958), tropical green mussel (Q9GZ70), Antarctic krill (A7VKE0), Portunus sanguinolentus (A1YYV6), Squilla oratoria (A5JVN3), North Pacific krill (A7VKE1), red swamp crayfish (C0LU07), brown shrimp (Q3Y8M6), crucifix crab (Q9N2R3), Japanese flying squid (Q6E216) and horned turban (Q7M3Y8). The land animal species included were bovine (Q5KR49), chicken (P04268), pig (P42639), rabbit (P58772) and Japanese quail (Q6LDU0, P58773). Other animal species included were the European common frog (P13105), African frog (Q01173) and German cockroach (Q9NG56). The program used for analysis was T-COFFEE version 7.71 (Notredame and others 2000).

2.3 Results and Discussion

2.3.1 The thermal stable protein banding pattern in cooked fish samples

Most of the soluble proteins in the fish extracts were heat labile and lost solubility after cooking (100°C for 15 min) (Fig. 3), although there were several major thermal stable proteins (130 kDa, 36 kDa and 12 kDa) in the cooked extracts (Fig. 3B). The protein around 36 kDa was clearly the dominant protein in all the cooked fish extracts tested. Comparing the 36 kDa protein among all the fish species tested, only the samples of yellowfin tuna and salmon showed species-specific characteristics. Unlike the other fish samples tested, which showed only a single protein band, two protein bands appeared around 36 kDa in yellowfin tuna. The protein band in the salmon sample had a slightly higher molecular weight than the equivalent bands in other fish species. The molecular weight of the 36 kDa protein in the salmon sample was also found to be similar to the molecular weight of the purified salmon tropomyosin.
Several fish proteins (130 kDa, 36 kDa and 12 kDa) in the cooked fish extracts withstood heating at 100 °C for 15 min (Fig. 3B), while most of the remaining proteins were degraded or denatured by heat. The molecular weight of the 12 kDa thermal-stable protein was similar to the molecular weight of fish parvalbumin (10–13 kDa) (Elsayed and Aas 1971). In addition, fish parvalbumin has been reported to be a thermal-stable allergenic protein in fish (Lindstrom and others 1996). Furthermore, the 12 kDa protein band in the SDS-PAGE gel (Fig. 3B) was relatively weak in both yellowfin tuna and salmon. A similar observation for fish parvalbumin was reported by Gajewski and Hsieh (2009). The anti-fish parvalbumin antibodies cross-reacted with most fish species tested, whereas these antibodies did not react with yellowfin tuna, salmon, swordfish (Gajewski and Hsieh 2009). Therefore, the 12 kDa protein is likely to be fish parvalbumin based on its thermal-stability, molecular weight and protein pattern.

One thermal-stable protein with a high molecular weight (130 kDa) was observed in the cooked fish extracts (Fig. 3B). There was no direct information with which to identify these proteins, but two fish proteins with high molecular weights of 120 and 240 kDa have been reported to be allergic proteins (Hamada and others 2001). Hamada and coworkers used a competitive ELISA with the sera from fish allergic patients to confirm the allergenicity of 120 and 240 kDa proteins in fish. The 120 kDa protein was reasonably attributed to correspond to the α-chain of collagen and the 240 kDa protein to the β-chain of collagen as a dimer of the α-chain. Hamada and others (2003a) went on to report that these proteins contain two collagen-specific amino acids, hydroxyproline and hydroxylysine, thus further confirming the allergenic protein identity as collagen. It is possible that the heat treatment partially degrades the proteins, thus affecting their molecular weight. Consequently, these two thermal-stable proteins among the fish samples tested could be collagen residues, aggregated proteins, or polymers of unknown proteins.

The dominant thermal-stable protein was a 36 kDa protein which appeared in all fish species tested (Fig. 3B). Besides the thermal-stability of this 36 kDa protein, the 36 kDa protein exhibited two species-specific patterns of the protein profile in cooked samples. These patterns were double bands in the yellowfin tuna sample and a high protein band in the salmon sample (Fig. 3B). Similar observations in fish tropomyosin have been reported by other groups. The SDS-PAGE of isolated tropomyosin from several fish species revealed that some fish species, for example bluefin tuna and pufferfish, have both α- and β-tropomyosin isoforms. Other fish
species, like swordfish and cod, had only the $\alpha$-form of tropomyosin in their skeletal muscle (Huang and Ochiai 2005; Heeley and Hong 1994). In addition, the results of Huang and Ochiai (2005) showed that the molecular weight of isolated salmon tropomyosin was slight higher than that of other fish species tested. A similar result was observed here: as Fig. 3B shows, the 36 kDa protein in salmon sample had a higher molecular weight than the 36 kDa protein in any of the other fish species.

2.3.2 Molecular weight migration of the 36 kDa protein in the presence of urea

One of the major characteristics of tropomyosin is the migration of molecular mass in the presence of urea in an electrophoretic gel (Sender 1971; Shanti 1993). The 36 kDa protein in cooked fish samples and the purified salmon tropomyosin were therefore analyzed by SDS-PAGE both in the absence (Fig. 4A) and in the presence of 6M urea (Fig. 4B). The molecular weight of the 36 kDa protein in all the fish samples tested shifted from 36 kDa to 50 kDa in the gel in the presence of urea as well as in purified salmon tropomyosin (Fig. 4B). To confirm that the shifted proteins were indeed the 36 kDa protein, the separated proteins were blotted with MAb 8F5 (Fig. 5). The result of the immunoblot showed the migration of the molecular weight of both the 36 kDa protein and the salmon tropomyosin in the presence of urea (Fig 5B). This molecular weight migration is due to the effect of the urea on the SDS binding of protein. Takagi and Kubo (1979) reported that $\alpha$-helical protein reduced the SDS binding by 50-70%. Because tropomyosin is an $\alpha$-helical protein (Woods 1976), the molecular weight of fish tropomyosin is changed by adding urea to the gel. In contrast, both the 36 kDa protein in all the fish samples tested and the salmon tropomyosin were recognized by MAb 8F5 (Fig 5). The weak antigenic protein band for yellowfin tuna was due to the relatively low antigenic protein content in the sample; although all the cooked fish samples were loaded in a fixed volume (10μl), the low antigenic content of the yellowfin tuna sample is because MAb 8F5 only recognizes one of yellowfin tuna’s tropomyosin isoforms.
2.3.3 Comparison of amino acid composition and sequence between the 36 kDa protein and fish tropomyosin

The amino acid composition and protein sequencing were performed to further characterize the 36 kDa protein and along with the fish tropomyosin. The 36 kDa protein was purified from the protein extract of cooked Atlantic salmon and the Atlantic salmon tropomyosin was also purified by the published method (Huang and Ochiai 2005). The results of SDS-PAGE revealed no obvious minor protein band except the 36 kDa protein and fish tropomyosin (Fig. 6). The immunoblot using MAb 8F5 further confirmed the purified protein to be the antigenic protein of MAb 8F5. The amino acid composition of the 36 kDa protein of salmon was analyzed along with the tropomyosin from salmon and the results compared with published references for salmon tropomyosin (Fig. 7). The amino acid composition was presented in terms of the percentage of the total for each amino acid. The results indicated that the amino acid composition of the 36 kDa protein was very similar to that of the purified fish tropomyosin as well as to the published reference for fish tropomyosin. To further characterize the 36 kDa protein at the molecular level, N-terminal protein sequencing was performed to obtain the amino acid sequence of the 36 kDa protein and purified fish tropomyosin. Initial attempts to sequence the 36 kDa protein without tryptic digestion were unsuccessful because the N-terminus of the 36 kDa protein was blocked, which confirmed the findings of Urbancikova and Hitchcock-DeGregori (1994), who reported that N-terminus of tropomyosin to be blocked by acetylation. After the 36 kDa protein and fish tropomyosin had been subjected to proteolytic digestion with trypsin, the digested protein fragments of the 36 kDa protein and fish tropomyosin were selected for protein sequencing and the results of protein sequencing analyzed with the blast program of Swiss Prot (Fig. 8). The amino acid sequences of the selected fragments of the 36 kDa protein and purified fish tropomyosin were the same. This sequence contained 12 amino acid residues (IQLVEEELDRAQ) and identically matched the corresponding amino acid sequence for the tropomyosin of salmon in the data base (Fig. 8). Based on these results, the fish 36 kDa protein is identified as fish tropomyosin.
2.3.4 The fish-specific region of the 36 kDa protein

A peptide array was employed to characterize the fish-specific region on the fish tropomyosin by using the fish-specific MAb 8F5 as the probe. The peptide array was prepared according to the amino acid sequence of Atlantic salmon tropomyosin (Swiss-Prot # Q91472) in the data base of Swiss-Prot. The peptides were coated on the cellulose membrane and had 10 amino acid residues with 3 amino acid residues overlapping for each peptide. This peptide array enabled the antibody to react with an extensive series of short peptides to localize the epitope of the antibody. The fish-specific MAb 8F5 was applied to react with the peptides on the membrane. The results showed that amino acid sequence “DLVALQ” was recognized by MAb 8F5 as its epitope (Fig.9). Because MAb 8F5 is fish-specific, it is important to further analyze the amino acid sequence of its epitope. The amino sequences obtained from the Swiss-Prot data base included 13 fish species, 5 land animal species, 19 shellfish species and 3 other animal species. The comparison was processed by the T-COFFEE program from the European Bioinformatic Institute (Notredame and others 2000) After comparing the tropomyosin sequences for the fish and non-fish species, part of sequence “DL” in the MAb 8F5 epitope was identified as being the fish-specific region (Fig.9). These results confirm the fish-specificity of MAb 8F5.

2.3.5 The 36 kDa thermal-stable protein as a suitable marker protein for fish detection

Currently, most immunoassays for the detection of food allergens have been developed using allergenic proteins as the marker proteins such as tropomyosin for shellfish detection (Fuller and others 2006). For example, an immunoassay was developed using fish allergenic protein, parvalbumin, to identify the presence of fin fish (Fæste and Plassen (2008). However, Gajewski and others (2009) found that the amount of the parvalbumin in cooked salmon, pollock and yellowfin tuna samples had been too small to be detected and had therefore lost its immunoreactivity to the anti-parvalbumin antibodies, rendering it unsuitable as a marker protein for fish detection. At present, no suitable marker protein to indicate the presence of fish in food has been reported.

This study investigated the immunoreactivity, molecular weight migration in urea gel, amino acid composition, and protein sequence of the 36 kDa thermal-stable protein in cooked
fish extracts. Our results confirmed that the 36 kDa protein is indeed fish tropomyosin. Because heating is a common method for cooking fish, a suitable marker protein for fish detection must be thermal-stable as well as uniformly distributed in fish muscle. Fish tropomyosin satisfies both these criteria, being a thermal-stable protein and uniformly distributed in fish muscle (Thys and others 2001). MAb 8F5 was also found to be within the conserved region in fish tropomyosin. Therefore, fish tropomyosin has the potential to serve as a suitable marker protein in an immunoassay for the detection of fish in food products.

2.4 Summary

This study identified the fish 36 kDa protein as fish tropomyosin based on its thermal-stability, similarity of molecular weight, migration of molecular weight and matched biological characteristics (amino acid composition and amino acid sequence). Fish tropomyosin is a ubiquitous protein uniformly distributed throughout the muscle of the fish, from head to caudal fin. Also, fish tropomyosin is abundant and dominant in cooked extracts among all the fish species tested. The conserved region of fish tropomyosin was revealed by fish-specific MAb 8F5. Therefore, tropomyosin was deemed a suitable marker protein to represent the presence of fish in food.
CHAPTER THREE

DEVELOPMENT OF A SANDWICH ELISA FOR THE DETECTION OF FISH MUSCLE

3.1 Introduction

Fish is known to be one of the major allergen foods that cause food allergic reactions in both children and adults. Sampson (2004) reported that fish were responsible for 0.1% and 0.4% of food allergies in US children and adults, respectively, and Bock and others (2001) reported that 1 in 32 deaths listed in a national registry of food-induced fatal anaphylaxis were caused by fish. To protect sensitized people from the often severe consequences of their food allergy, the Food Allergen Labeling and Consumer Protection Act (FALCPA) requires that any protein-containing ingredient derived from the eight major allergen foods must be declared in the ingredient statement on the label of foods (Public Law 108-282). Besides the allergy concerns related to fish in human food, fishmeal, a common animal feed ingredient, has been banned for use in animal feed in Japan, Australia, and European Union to reduce the risk of BSE (EC 999/2001). Although no evidence had been found to indicate that fishmeal could cause BSE, fishmeal may be accidentally contaminated by banned meat tissue during processing, packing and storage.

At present, the only officially approved method for fish detection in animal feed is microscopy, which requires specially trained personnel and expensive equipment (Commission Directive 2003/126/EC). Currently, no assays have yet been developed that are capable of detecting fishmeal in either food or feed. Although a number of antibodies to detect fish species such as grouper, wreck fish, Nile perch (Asensio and others 2003), red snapper (Huang and others 1995) and pangasius catfish (Hsieh 2009) have been reported, none are able to cover all fish species. Fæste and Plassen (2008) developed an polyclonal antibody based sandwich ELISA to detect a major fish allergenic protein, parvalbumin, in food but the results showed non-specific reactivity and the assay was not able to detect parvalbumin in all the fish samples tested. The
research described in this chapter was therefore designed to address this lack by developing a sandwich ELISA capable of detecting fish and fish products in food.

The major advantages of sandwich ELISA are that no purification is required for the sample preparation and its strong commercial potential. As described earlier in Chapter 2, fish tropomyosin was identified as a suitable marker protein to indicate the presence of fish based on its common distribution among fish muscle, thermal-stability and its fish-specificity. Therefore, fish tropomyosin was selected for use as the target protein for the development of a fish-specific sandwich ELISA.

MAb 8F5 had previously been developed in our laboratory for the development of the fish-specific sandwich ELISA. In the research described in the last chapter, MAb 8F5 cross-reacted with cooked samples of all 55 of the fish species tested and was recognized as fish tropomyosin, the antigenic protein. Because sandwich ELISA requires a pair of antibodies, another antibody was needed to pair with MAb 8F5. To obtain a matched pair of antibodies, the pooled crude protein extracts from ten fish species (Atlantic salmon, yellowfin tuna, swordfish, black grouper, tilapia, red snapper, amberjack, basa, catfish, and perch) were used as the immunogen to develop suitable polyclonal antibodies. This chapter describes the characterization of the polyclonal antibody and how a matched pair of antibodies was selected with which to develop the assay. After optimization, the assay was validated by examining its species specificity against raw and cooked fish samples and immunoreactivity with various processed fish products, as well as the limit of detection and precision of the assay.

3.2 Materials and Methods

3.2.1 Materials

Tris-buffered saline, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl (pH 8.8), N,N,N’,N’-tetra-methyl ethylenediamine (TEMED), Precision Plus Protein Kaleidoscope Standards, 30% acrylamide/bis solution, Tris/glycine buffer, 10 × Tris/glycine/SDS buffer, supported nitrocellulose membrane (0.2 μm), and thick blot paper were obtained from Bio-Rad Laboratories Inc. (Hercules, CA). Hydrogen peroxide, horseradish peroxidase conjugated goat
anti-mouse IgG (Fc specific), ABTS (2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid), and β-mercaptoethanolwere and HRP-streptavidin were purchased from Sigma-Aldrich Co. (St. Louis, Mo., U.S.A.). Bromophenol blue sodium salt was purchased from Allied Chemical Corp. (N.Y., U.S.A.). Protein Assay Kit II was purchased from Bio-Rad. Sodium chloride (NaCl), sodium phosphate dibasic anhydrous (Na₂HPO₄), sodium phosphate monobasic anhydrous (NaH₂PO₄), bovine serum albumin (BSA), sodium bicarbonate (NaHCO₃), sodium carbonate (Na₂CO₃), citric acid monohydrate, sodium dodecyl sulfate, Tween 20, and all other chemicals, reagents, syringe filters (0.45 μm Millipore, Millex R-HV), Whatman No.4 filter paper, and 96-well polystyrene microplates (Costar 9018) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). All solutions were prepared using distilled deionized pure water (DDI water) from a NANO pure Diamond ultrapure water system (Barnstead Intl., Dubuque, Iowa, U.S.A.). The homogenizer used was the ULTRA-TURRAX T25 basic homogenizer (IKA Works, Inc., Wilmington, NC). The SmartSpec 3000 UV spectrophotometer and the Model 1575 ImmunoWash microplate washer were supplied by Bio-Rad and the PowerWave XS microplate reader by Bio-Tek Instruments (Winooski, VT). The materials purchased from Pierce were NHS-activated agarose resin (Pierce 26197) and Sulfo-NHS –biotin Pierce21217). All chemicals and reagents were analytical grade. The fish samples were provided by government agencies or purchased from seafood retail chains and domestic seafood markets. The shellfish and land animal samples were purchased from local stores and seafood markets.

3.2.2 Methods

3.2.2.1 Production of polyclonal antibody for fish detection

The immunogen for the development of the polyclonal antibody are partially purified fish tropomyosin. The preparation of fish tropomyosin generally followed the published method (Huang and Ochiai 2005). To prepare the immunogen, equal amounts of fresh fish muscle from each fish species were taken from Atlantic salmon, yellowfin tuna, swordfish, black grouper, tilapia, red snapper, amberjack, basa, catfish, and perch. The fresh fish muscle was minced and placed in a beaker then heated in a water bath (100°C) for 15 min. Cooked fish samples were homogenized for 1 min at 13,000 rpm with 5 vol. (v/w) 0.15M NaCl solution and then centrifuged (20,000xg at 4°C, 30min) and filtered (Whatman No.4). The clear supernatant was subjected to isoelectric precipitation at pH 4.5 with 1 N HCl. After centrifugation at 20,000xg for
20 min the pellet was brought to pH 7.6 with 1 N NaOH, and subjected to ammonium sulfate fractionation, yielding a 50–60% saturated fraction. These procedures were repeated twice, and the final fraction dialyzed against PBS. The protein concentration of the resulting protein extracts were determined by Protein Assay Kit II (Bio-Rad) according to the method of Bradford. The immunogen (1.2 mg/ml) was diluted in 0.5 ml sterile saline solution and mixed with adjuvant. A polyclonal antiserum was raised in New Zealand rabbits. The 1st boot injected 200 µg protein with Freund’s complete adjuvant and 100 µg protein with Freund’s incomplete adjuvant for later booting procedures. After the second and third boots, blood from the rabbits was collected at the 7th week. The IgG in the pooled serum was collected by Protein-A affinity chromatography and diluted in PBS. The total protein concentration was determined by the Lowry method (Lowry and others 1951). The production of this polyclonal antibody was performed by Abbiotec (San Diego, CA, USA). The purified IgG (5 mg/ml) was stored in aliquots at -80°C until use. For convenience, the polyclonal antibody is referred to as PAb B hereafter.

3.2.2.2 Depletion of polyclonal antibodies

To address the issue of non-specific cross-reactivity, crude protein extracts of cooked pork and chicken were prepare for the depletion of PAb B using an antibody depletion method modified from the published method (Tyler and others 1989). The fresh muscle from pork or chicken was minced and placed in a beaker then heated in a water bath (100°C) for 15 min. Cooked fish samples were homogenized for 1 min at 13,000 rpm with 5 vol. (v/w) 0.15M NaCl solution and then centrifuged (10,000xg at 4°C, 30min) and filtered (Whatman No.4). A 4 ml sample of the undiluted crude extract was then mixed end-over-end with 330 mg of NHS-activated agarose resin (26197, Pierce) in a tube at 4°C overnight. The NHS-activated resin was packed into a spin column (26198, Pierce) and washed 10 times by 4 ml PBS. After washing, the NHS-activated resin was quenched by 4ml 1M Tris buffer at room temperature for 20 min. After a further washing with 4 ml PBS, 2 ml purified polyclonal antibody (5 mg/ml) in PBS was loaded into the column and mixed end-over-end at 4°C overnight. After mixing, the spin column was centrifuged at 1000xg for 1 min at 4°C. The flow-through antibodies in the solution were then collected. The depletion procedures were repeated twice to eliminate the non-species specificity of the PAb B. After depletion, the PAb B was named PAb BD. The concentration of
PAb BD (1.2 mg/ml) was determined by a UV spectrophotometer at 280 nm. The PAb BD was stored in aliquots at -80°C until use.

### 3.2.2.3 Biotinylation of the antibodies

The method used for the biotinylation of the antibodies was modified from the published method (Gretch and others 1987). The PAb BD was conjugated with biotin using Sulfo-NHS – biotin (21217, Pierce) according to the manufacture’s protocol. The 3.7 µl of 10 mM biotin was added into 500 µl of the antibody solution (1 mg/ml). The reaction was carried out at 4°C for 2 hours and resulted in 4~6 biotin groups per IgG. The extra biotin in solution was excluded by dialysis in PBS overnight. The concentration of the biotin-conjugated PAb BD was determined by a UV spectrophotometer at 280 nm. The biotin-conjugated PAb BD was stored in aliquots at -80°C until use.

### 3.2.2.4 Sandwich ELISA procedure

The ninety six well flat microplate was coated overnight at 4°C with 100 µl/well of PAb B diluted in PBS buffer to 5 µg/ml. Then, the plate was washed with PBS containing 0.005% Tween-20 (PBST, pH 7.6) three times by using a microplate washer. All the washing steps in the sandwich ELISA were the same. After washing, the unsaturated binding sites on the surface of the microplate were blocked by the PBS containing 1% BSA at 37°C for 1 hour. After washing, the samples at 1:2 dilution in PBS were added on the microplate at 37°C for 2 hours. After washing, bound proteins were detected by adding 100 µl/well of biotinylated PAb BD diluted to 1µg/ml and incubated at 37°C for 1 hour. Following the washing, the microplate was incubated at 37°C for 1 hour with 100 µl/well of HRP-streptavidin conjugated at 1:3,000 dilution. After a final washing, 100µl of 0.4 mM ABTS substrate solution [0.22 mg/ml of ABTS in 50 mM phosphate-citrate buffer (50 mM Na₂HPO₄ and 25 mM citric acid, pH 5.0) containing 0.15 µl/ml of H₂O₂] was added to each well for color development at 37°C. After 20 min, the color development was stopped by stop solution (0.2M citric acid). Absorbance was read at 415nm using a microplate reader.
3.2.2.5 Fish sample extraction

The physiological characteristics of fish muscle protein are affected by both the species and habitat of fish (Huang and Ochiai 2005). To control for (or minimize the effect of) these factors, common food fish species from fresh water and salt water were selected as the samples. Fresh fish muscle was minced and placed in a beaker then heated in a water bath (100°C) for 8 min. Cooked fish samples were homogenized 1 min at 13,000 rpm with 5 vol. (v/w) 0.15M NaCl solution and then centrifuged (10,000xg at 4°C, 30min) and filtered (Whatman No.4). The protein concentration of these protein extracts was determined by Protein Assay Kit II (Bio-Rad) according to the method of Bradford and the extracts were stored at -20°C until use.

3.2.2.6 Preparation of spiked samples

To evaluate the sandwich ELISA, three fish species (pollock, whiting and basa) were selected for the preparation of the spiked samples. One gram of freshly minced fish muscle was mixed with nine grams of fresh crab meat to make 10% (w/w) adulterated samples. For the preparation of the cooked spiked sample, the mixture of minced raw fish and crab meat in the beaker was covered by aluminum foil and heated in a water bath (100°C) for 8 min. After cooking, 5 vol. (v/w) 0.15M NaCl solution was added to the beaker. For the preparation of the raw sample, 5 vol. (v/w) 0.15M NaCl solution was added to a beaker containing the mixture of minced raw fish and crab meat. Both raw and cooked samples were homogenized for 1 min at 13,000 rpm and then centrifuged (10,000xg at 4°C, 30min) and filtered (Whatman No.4). Extracts of unadulterated raw and cooked samples (crab meat) were prepared in the same manner. Because it was difficult to handle extremely small amounts of fish muscle for the preparation of spiked samples at very low adulteration levels, the spiked samples were prepared by using 10% (w/w) adulterated sample extracts to achieve the required sample homogeneity. After extraction, the adulterated samples were prepared by diluting the 10% (w/w) adulterated sample extract with unadulterated crab meat extract at the same levels as the adulterated meat samples at five adulteration levels: 0.1ppm, 1 ppm, 10 ppm, 100 ppm and 1000 ppm. The crab meat containing no fish (0% sample) served as the negative control, and 100% ground fish samples as the
positive controls. The limit of detection (LOD) was defined as the smallest adulation level that could be significantly differentiated from the background (0% adulteration) level of the assay.

### 3.2.2.7 Validation of the sandwich ELISA

The species specificity of the sandwich ELISA was examined for 64 fish species, 4 shellfish species, 8 land animal species and 4 common food additive proteins (Appendix C). The collected samples were stored at -80°C prior to sample preparation. To prepare the protein extracts, the fresh muscle tissue was minced and placed in a beaker. For the preparation of cooked samples, the minced raw muscle in the beaker was covered by aluminum foil and heated in a water bath (100°C) for 8 min. After cooking, 5 vol. (v/w) 0.15M NaCl solution was added to the beaker. For the preparation of raw sample, 5 vol. (v/w) 0.15M NaCl solution was added to the beaker with minced raw muscle. Both raw and cooked samples were homogenized 1 min at 13,000 rpm and then centrifuged (10,000xg at 4°C, 30min) and filtered (Whatman No.4). The protein extracts were stored at -20°C until use.

To study the effect of the food processing on the immunoreactivity of fish, fish products with various processes (salting, smoking, canning) and fishmeal were purchased from local stores. The extraction procedure for the fish products and fishmeal was the same as previously described. Raw fish product such as salted mackerel was extracted both with and without heat treatment (100°C, 8 min). The “fully cooked” or “ready to eat” fish products such as canned tuna and smoked salmon were extracted without heat treatment. The protein extracts were stored at -20°C until use.

To evaluate the reproducibility of the assay, the intra-assay variability represented by coefficient of variation (%CV) and inter-assay variability were determined using spiked samples in order to identify the detection limits. The intra-assay CV among the determinations within a plate was measured by analyzing at least 3 replicates of each sample in the assay. The inter-assay CV was calculated by analyzing 3 replicates of each sample carried out on 2 different days.
3.2.2.8 Statistical analysis

All the experiments of the sandwich ELISA were performed triplicate and repeated in different day. One-way analysis of variance (ANOVA) was performed using SPSS software (12.0 for Windows; SPSS Inc., Chicago, Ill., U.S.A.) to reveal differences among treatments. Dunnett’s post test was used to determine the detection limit by comparing differences in the ELISA readings between the baseline and the laboratory-adulterated samples. All data were obtained at least in triplicate and experiments were repeated twice. Statistical significance was accepted at $P = 0.05$.

3.3 Results and Discussion

3.3.1 Antibody characterization

To develop a fish-specific sandwich ELISA, we developed a polyclonal antibody (PAb) by immunizing rabbits with partially purified fish tropomyosin from ten fish species: Atlantic salmon, yellowfin tuna, swordfish, black grouper, tilapia, red snapper, amberjack, basa, catfish, and perch. This antibody is referred to here as PAb B.

The specificity of PAb B was analyzed by indirect ELISA, coating 2 µg/well of each of the protein extracts from fish, land animals, shellfish and food additives (Fig. 10). The results of the indirect ELISA showed that PAb B reacted with both raw and cooked fish extracts. The absorbance of PAb B with cooked fish samples (cod and catfish) was around 2.2~2.4, markedly higher than raw fish samples (0.6~0.7) (Fig. 10). PAb B did not cross-react with either raw non-fish samples (land animals, shellfish) or food additives (Fig. 10) and there was no immunoreaction between pre-immune serum and cooked fish samples (Fig. 10). Although PAb B did not cross-react with cooked shellfish samples, it did cross-react with cooked samples of pork, turkey and chicken (Fig. 10). The non-specific immunoreactivity of the PAb B was then depleted by NHS-activated chromatography with the immobile phase of chicken and pork protein extracts. After depletion, the PAb B no longer cross-reacted with the cooked samples of chicken, turkey, pork and other animals (Fig. 11). In this study, the depleted PAb B is referred to as PAb BD.
The binding characteristics of PAb BD were determined on immunoblot using purified salmon tropomyosin and cooked extracts of six fish species (cod, red snapper, red grouper, tilapia, basa and shark), including bony and cartilaginous fish from both fresh and salt water. PAb BD recognized both the 36 kDa protein among the cooked fish samples tested and the purified salmon tropomyosin (Fig. 12). The results of immunoblot verified that the antigenic protein of the PAb BD is indeed fish tropomyosin.

At present, the only available antibody for fish detection was developed by Fæste and Plassen (2008). However, its utility is limited, as although the polyclonal antibody was raised against purified cod parvalbumin it also recognizes several non-specific proteins, fails to react to raw samples of Northern pike and anchovy, and cross-reacts to squid samples (Fæste and Plassen 2008). Furthermore, the amount of parvalbumin in certain fish species, including tuna and salmon, is actually very low; Gajewski and Hsieh (2009) reported that anti-fish parvalbumin antibodies cross-reacted with most fish species tested but did not react with yellowfin tuna, salmon, or swordfish. As a result, fish parvalbumin is not a suitable marker for the presence of fish. As previous described in Chapter 2, fish tropomyosin is the dominant thermal-stable protein in cooked fish extract and is commonly distributed among different fish species. In this study, the PAb B was developed using partially purified fish tropomyosin from cooked fish extracts and the immunogen pooled the fish tropomyosin from ten salt and fresh water fish species to enhance the cross-reactivity of the PAb B to a variety of food fish species. Although the PAb B showed cross-reactivity with cooked chicken and pork samples, it became a fish-specific antibody after depleting its non-specific immunoreactivity.

3.3.2 Selection of the antibodies for the sandwich ELISA for fish detection

Sandwich ELISA is performed by using a pair of antibodies to capture a target protein (capture antibody) and then detect that captured target protein (detection antibody) (Fig. 13). In sandwich ELISA, each antibody is required to recognize a different and not overlapping epitope on the target protein. Here, the fish-specific antibody, MAb 8F5, is a candidate antibody for the development of a sandwich ELISA. PAb BD was initially developed to pair with MAb 8F5 to serve as either the detection or the capture antibody. For the selection of the detection antibody
and capture antibody to be utilized in the sandwich ELISA, MAb 8F5, PAb BD, PAb B were tested in various pairs. Although MAb 8F5 was a candidate antibody, the absorbance was very low when MAb 8F5 paired with PAb BD (Fig. 14). These results indicate that PAb BD may compete with the epitope of MAb 8F5 on the target protein. Unlike a monoclonal antibody from a single clone, a polyclonal antibody represents the antibodies from multiple clones and is therefore specific to more than one epitope on the antigenic protein. Since the fish-specific regions on fish tropomyosin are limited, this increases the likelihood of epitope competition between MAb 8F5 and PAb BD. Of the combinations tested, the highest absorbance was obtained using PAb B as the capture antibody and PAb BD as the detection antibody. Because PAb B contains more antibodies specific to the consensus regions, it experienced less competition for the conserved regions with the PAb BD. Therefore, PAb B and PAB BD were selected as the capture antibody and detection antibody, respectively.

### 3.3.3 Optimization of the sandwich ELISA

A two dimensional checker board experimental design for the optimization of the sandwich ELISA was performed step by step to select the optimal concentrations of the capture antibody and detection antibody (Table 1). Because there are so many food fish species it is difficult to optimize the sandwich ELISA for every fish species. Cooked cod extracts were chosen for optimizing the sandwich ELISA, because cod was the first and remains the most commonly reported fish species causing allergic reactions (Aas and Jebsen 1967). The cooked cod extracts was diluted 1:2 in PBS. Seven different dilutions (1:2,000~1:160,000) were used for the coating of the capture antibody PAb B (6 mg/ml), and the detection antibody PAb BD (1 mg/ml) was diluted into four dilutions (1:1,000~1:10,000). The results showed that 1:2,000 for the capture antibody PAb B and 1:1000 for the detection antibody PAb BD produced the best result (OD value: 1.67) (Table 1). Later in the optimization of the sample dilution, the amount of capture antibody was increased to 1:1,000, giving an even better result (OD value: 2.3) (Fig. 15). Therefore, the optimized condition for the sandwich ELISA was chosen as 1:1,000 for the capture antibody PAb B and 1:1,000 for the detection antibody PAb BD.
To optimize the sample dilution factor, both raw and cooked fish samples were diluted into seven different dilutions (1:2~1:80). The fish samples used for this test were from cod and yellowfin tuna which tend to have lower protein concentrations than other fish species after heat treatment. The results showed no significant difference between the seven dilutions in raw samples of cod and yellowfin tuna (Fig. 15A). Although no significant difference was observed for the cooked cod samples in any of the dilutions, the overall trend of absorbance of cooked yellowfin tuna samples decreased from 1:2 to 1:80 as the samples became more diluted (Fig. 15B). Based on these results, a sample dilution of 1:2 was selected for the sandwich ELISA. The optimum color development was 20 min to obtain the best sensitivity and low background.

3.3.4 The species specificity of the sandwich ELISA

The examination of the species specificity of the sandwich ELISA was performed with 64 common food fish species, 4 shellfish species, 8 land animal species and 4 food additives in both raw and cooked. The results showed that the sandwich ELISA was capable of detecting all 64 of the fish species tested in both raw and cooked samples without any cross-reaction with the samples of shellfish, land animals and food additives (Fig. 16 & Fig. 17). The highest absorbance in the raw fish samples tested was 2.05 (pollock) and the lowest was 0.67 (shark) (Fig. 16). The absorbance of most of the raw fish samples was between 1.4~1.8 (Fig. 16), while the absorbance of most of the cooked fish samples was between 1.8~2.0, higher than the raw fish samples (Fig. 17). In the cooked fish samples, the highest absorbance was 2.21 (swordfish) and lowest was 1.24 (rainbow trout) (Fig. 17). In addition, there was no cross-reactivity with either raw or cooked samples of shellfish, land animals or food additives. These results indicate that the sandwich ELISA developed for this project is indeed highly specific to fish species without any cross-reaction with non-fish species.

The 64 fish species used to examine the species specificity of the sandwich ELISA included most of the common food fish species from both salt water and freshwater sources such as grouper, snapper, salmon, tuna, swordfish, amberjack, mahi-mahi, mackerel, flounder, tilapia, catfish and bass. The fish samples included not only bony fish species but also cartilaginous fish species (shark). However, it was not possible to collect samples of every single food fish species
in the world to be tested by the sandwich ELISA, although the number of fish species tested here was far higher than any previously published assay, the most comprehensive of which reported cross-reactions between 20 of the 32 fish species tested (Fæste and Plassen 2008). In this study, the sandwich ELISA showed strong immunoreactivity to most of the fish species tested in both raw and cooked samples. However, the sandwich ELISA may not have strong reactivity to the fish species belonging to the *Siluriformes* family (basa, tra, catfish, channel catfish) and *salmonidae* family (Atlantic salmon, sockeye salmon, Coho salmon, rainbow trout) (Fig. 16 & Fig. 17). This could be due to the natural differences in the protein sequence of the tropomyosin in these fish species.

Generally, the new sandwich ELISA is capable of recognizing all of the raw and cooked fish samples tested. Furthermore, one great advantage of this sandwich ELISA is its ability to detect cartilaginous fish species, which are currently not recommended for human consumption because of their high mercury levels (Hightower and Moore 2003). At present, there is no method available for the detection of cartilaginous fish species in food. Also, the immunoassay for the detection of fish developed by Fæste and Plassen (2008) showed some cross-reactivity with squid. Therefore, the new sandwich ELISA was also tested with squid, shrimp, scallop and crab and the results showed no cross-reaction with shellfish species. Based on these results, the fish-specific sandwich ELISA has been successfully developed for the detection of bony and cartilaginous fish.

3.3.5 The detection of various processed fish products by the sandwich ELISA

The immunoreactivity of selected salted, smoked and canned fish products were examined by the new sandwich ELISA along with unprocessed fish samples of the same species. The fish products that needed to be cooked before consumption were examined in both the raw and cooked. If the fish products claimed to be “fully cooked” or “ready to eat” on the label of the package, these products were compared with either cooked or raw fish samples.

The results showed that the salting process has a significant effect on both raw and cooked pompano samples and on cooked mackerel samples (Fig. 18). In general, a neutral solution with ionic strength from 0.3 to 1.0 was considered suitable for the extraction of myofibrillar proteins.
In contrast, sarcoplasmic proteins were extracted in solutions with a sodium chloride concentration of less than 0.15 M (Scopes 1970). In this study, the samples were extracted by 0.15 M sodium chloride. This extra salt may increase the extractability of the protein, but the results obtained may vary due to the characteristics of the different fish species tested.

The smoking process did not appear to affect the immunoreactivity of raw mackerel, but the immunoreactivity was significantly decreased in smoked salmon and cooked smoked mackerel (Fig. 19). However, it is important to note that the actual procedures used for smoking could vary widely among the different manufacturers. There are two major smoking methods for smoked fish: hot-smoking (90°F for the first 2 hours; 150°F for additional 4–8 hours); and cold-smoking (80-90°F for 1-5 days). Some commercial smoked fish products are produced by soaking fish in smoke-flavored solutions. Therefore, it is difficult to investigate the major effect of the smoking process of fish products. However, several studies have indicated that muscle proteins are degraded by proteolysis after the post-mortem stage (Jasra and others 2001; Michalczyk and Surówka 2007). Since fish subjected to cold-smoking processing may be held at 80-90°F for 1-5 days, the target proteins may be degraded by proteolysis.

The canning process clearly decreased the immunoreactivity of canned tuna and canned salmon (Fig. 20). The severe heat treatment involved in the canning process may cause most of the proteins in the fish to become degraded. Bernhisel-Broadbent and others (1992b) reported that the allergenicity of tuna and salmon was minimized in both canned tuna and canned salmon. In their results, SDS-PAGE and immunoblot using the sera of fish allergic patients demonstrated that the allergic proteins had been significantly degraded in canned tuna and canned salmon. However, the new sandwich ELISA was still able to detect sufficient remaining target proteins in the canned fish products tested to give a positive result (Fig. 20). Fishmeal usually undergoes multiple processes, including rendering, drying, grinding and autoclaving, all of which combine to degrade most of the proteins. The epitopes on the target protein may be destroyed, and hence the immunoreactivity to the antibodies. Although the absorbance of the fishmeal was around 0.9 (Fig. 20), the sample of fishmeal was still readily detected by the new sandwich ELISA. Generally, the sandwich ELISA showed its ability to detect fish even after a range of common food processes and the processing involved in preparing animal feed.
Since the immunoreactivity of the target protein is very different among different fish species, it is difficult to determine accurate quantities for the target protein by using the target protein of a single fish species as the standard. Therefore, we attempted to develop a qualitative assay for the fish detection in food products. However, although the immunoreactivity of the antigenic protein in the samples was affected by the variety of fish species and common food and feed processing, the assay is clearly capable of performing satisfactory qualitative detection of both raw and cooked fish samples and fish products.

3.3.6 Limit of detection (LOD) of the sandwich ELISA

The LOD of the sandwich ELISA inevitably varies depending on the amount of target protein present in the various fish species. Therefore, testing of the LOD of the sandwich ELISA was based on a realistic real world case. Surimi is a fish product made using a fish muscle protein concentrate. Pollock and whiting are the major fish species used for its production, and surimi is usually used to produce imitation crab meat. Because the price of crab is much higher than imitation crab meat, imitation crab meat is often used to substitute for real crab meat in products such as stuffed crab and crab cake. Several other white muscle fish species such as basa, which have soft and flaky muscle, may be also be used to substitute for crab meat. Therefore, the LOD of the sandwich ELISA was tested using different levels of spiked pollock, whiting and basa in crab meat (Fig. 21). The fish samples were prepared in 10% (w/w). Extracts of the 10% sample were diluted into five different dilutions (1000 ppm, 100 ppm, 10 ppm, 1 ppm and 0.1 ppm) with crab sample extracts (v/v). The LOD of the raw samples was found to be 1 ppm for whiting and 0.1 ppm for pollock and basa (Fig. 21A). The LOD for the cooked pollock, whiting and basa samples was 0.1 ppm (Fig. 21B). Currently, the range of LOD in most commercial immunoassay products for the detection of food allergens is 0.5 ppm to 25 ppm; the LOD of the only commercial DNA-based assay for the fish detection is 10 ppm (Appendix C). These results show that the new sandwich ELISA is more sensitive than not only most commercial immunoassays for food allergens but also the DNA-based assay for fish detection. However, it is important to bear in mind that the actual LOD of the sandwich ELISA depends on the amount of target protein in the fish species tested.
For the determination of the reproducibility of the sandwich ELISA, the six sets of different laboratory spiked samples (1000 ppm–0.1 ppm) were analyzed and the value of coefficient of variations (CVs) were determined (Table 2 & Table 3). The intra-assay variability measured the variation between replicates of the same assay and the inter-assay variability measured the variation between assays performed on different days. The intra-assay CVs of the sandwich ELISA ranged from 0.28~8.88 % and the inter-assay CVs ranged from 0.93~9.28%. The CVs of both the intra-assay and inter-assay of the new sandwich ELISA were lower than the FDA’s acceptable CV (15%) (FDA 2001). The false-positive and false-negative rates were determined in the experiments on species specificity, fish products and the limit of detection (Table 4). The rate of false-positives was 0%. A total of 1380 samples containing fish protein were determined as positive by the assay. The rate of false-negatives was also 0%; a total of 324 samples without fish protein were all determined to be negative (Table 4).

3.3.7 Limitations of the assay and directions for future research

A new assay was successfully developed to detect both raw and cooked extracts of 64 fish species tested, which included most of the common food fish species consumed in the US. However, there is a wide variety of food fish species and fish products around the world and it was not possible to include them all. Furthermore, the amount of tropomyosin could be different for different growth stages of animals (Hosoya and others 1989). Both these factors may affect the sensitivity of the assay and future studies should go on to test a wider range of fish species and fish products, and to test the tropomyosin content in fish at different growth stages.

3.4 Summary

In this study, a fish-specific sandwich ELISA for the qualitative determination of fish in food was successfully developed. The assay was shown to be capable of recognizing all 64 of the fish species tested without any cross-reaction with shellfish, land animals or food additives. The validation included the detection of various processed fish products and a measurement of the limit of detection of three different fish species in crab meat. The assay was shown to be capable
of detecting various fish products (salted, smoked, canned) and fishmeal. The LOD of fish in crab meat was 0.1 ppm for both cooked and raw fish samples. The assay exhibited low intra-(%CV ≤ 8.9%) and inter-assay variability (%CV ≤ 9.4%). Overall, the assay has the potential to be a useful tool for both quality control during food production and for law enforcement by governmental agencies.
CHAPTER FOUR

CONCLUSIONS

The 36 kDa thermal-stable protein recognized by fish-specific MAb 8F5 was identified to be fish tropomyosin. The 36 kDa protein and fish tropomyosin are both thermal-stable proteins and have similar protein patterns on the SDS-PAGE. The molecular weight of the 36 kDa and fish tropomyosin shifted to 50 kDa in the presence of urea in the SDS-PAGE. In addition, the 36 kDa and fish tropomyosin had similar the amino acid composition and had identical protein sequence (12 amino acid residues). Based on these results, the 36 kDa antigenic protein of MAb 8F5 is likely to be fish tropomyosin.

A suitable marker protein for the presence of fish in food products and animal feed must be thermal-stable to withstand the processes commonly used in the food and feed industries. Fish tropomyosin is not only a thermal-stable protein but is also ubiquitous, being evenly distributed in all fish muscle tissues. A sandwich ELISA was therefore developed for the detection of fish tropomyosin as target protein to represent the presence of fish in food. After depleting its non-specific cross-reactivity, the PAb BD developed in this research was shown to be specific to fish. However, both PAb BD and PAb B were found to compete for the same epitope of MAb 8F5. The sandwich ELISA was thus developed using PAb B as the capture antibody and PAb BD as the detection antibody.

The optimized sandwich ELISA reacted to all 64 of the fish species tested for this study in both raw and cooked without any cross-reaction with shellfish, land animals and food additives. The optimized assay was also shown to be capable of detecting fish products after processes such as salting, smoking and canning. The assay exhibited good performance, with a low intra- (%CV ≤ 8.9%) and inter-assay variability (%CV ≤ 9.4%). The limit of detection of the assay was found to be 0.1 ppm for both raw and cooked pollock and whiting in crab meat. This polyclonal antibody based sandwich ELISA is therefore a suitable assay for the qualitative detection of fish muscle protein in food products and animal feed to improve food safety.
Table 1 Optimization of the capture and detection antibodies of the sandwich ELISA. The concentration of PAb B was 6 mg/ml and the concentration of PAb BD was 1 mg/ml. Samples consisted of cooked cod extracts diluted 1:2 in PBS. The absorbance was measured at 415 nm.

<table>
<thead>
<tr>
<th>Absorbance 415 nm</th>
<th>Detection Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAb BD 1:1000</td>
</tr>
<tr>
<td>PAb B 1:2000</td>
<td>1.673</td>
</tr>
<tr>
<td>PAb B 1:5000</td>
<td>0.435</td>
</tr>
<tr>
<td>PAb B 1:10000</td>
<td>0.218</td>
</tr>
<tr>
<td>PAb B 1:20000</td>
<td>0.163</td>
</tr>
<tr>
<td>PAb B 1:40000</td>
<td>0.159</td>
</tr>
<tr>
<td>PAb B 1:80000</td>
<td>0.110</td>
</tr>
<tr>
<td>PAb B 1:160000</td>
<td>0.117</td>
</tr>
</tbody>
</table>
Table 2 The inter- and intra- coefficient of variation (CV) of the sandwich ELISA for raw spiked samples.

<table>
<thead>
<tr>
<th>CV (%)</th>
<th>Inter-assay</th>
<th>Intra-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>Day 1</td>
</tr>
<tr>
<td>Pollock in crab</td>
<td>1000 ppm</td>
<td>2.97</td>
</tr>
<tr>
<td></td>
<td>100 ppm</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>10 ppm</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>0.1 ppm</td>
<td>3.32</td>
</tr>
<tr>
<td>Whiting in crab</td>
<td>1000 ppm</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>100 ppm</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>10 ppm</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>5.15</td>
</tr>
<tr>
<td></td>
<td>0.1 ppm</td>
<td>6.18</td>
</tr>
<tr>
<td>Basa in crab</td>
<td>1000 ppm</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>100 ppm</td>
<td>9.18</td>
</tr>
<tr>
<td></td>
<td>10 ppm</td>
<td>4.28</td>
</tr>
<tr>
<td></td>
<td>1 ppm</td>
<td>8.32</td>
</tr>
<tr>
<td></td>
<td>0.1 ppm</td>
<td>5.37</td>
</tr>
</tbody>
</table>

*a* Each sample was analyzed three times a day, and the assay was repeated on two different days.

*b* The pooled standard deviation (sp) was calculated using equation 1. The pooled mean (Xp) was calculated by substituting individual means for variances in equation 1. The intra-assay and inter-assay CVs were calculated using equation 2 (Grotjan and Keel 1996).

Equation 1

\[
s_p = \sqrt{\frac{\sum_{l=1}^{n} (df_l \times s_l^2)}{\sum_{l=1}^{n} df_l}}
\]

Where:

- \( s \): individual standard deviation (SD)
- \( df \): degrees of freedom (n-1, n: sample size of individual experiment)

Equation 2

\[
CV(\%) = (sp/Xp) \times 100
\]
Table 3 The inter- and intra-coefficient of variation (CV %) of the sandwich ELISA for cooked spiked samples.

<table>
<thead>
<tr>
<th>CV (%)</th>
<th>Inter-assay</th>
<th>Intra-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>Pollock in crab</td>
<td></td>
</tr>
<tr>
<td>1000 ppm</td>
<td>0.93</td>
<td>1.16</td>
</tr>
<tr>
<td>100 ppm</td>
<td>1.68</td>
<td>1.87</td>
</tr>
<tr>
<td>10 ppm</td>
<td>6.58</td>
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</tr>
<tr>
<td>1 ppm</td>
<td>5.03</td>
<td>2.67</td>
</tr>
<tr>
<td>0.1 ppm</td>
<td>9.28</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>Whiting in crab</td>
<td></td>
</tr>
<tr>
<td>1000 ppm</td>
<td>7.05</td>
<td>0.73</td>
</tr>
<tr>
<td>100 ppm</td>
<td>4.18</td>
<td>0.51</td>
</tr>
<tr>
<td>10 ppm</td>
<td>3.40</td>
<td>0.92</td>
</tr>
<tr>
<td>1 ppm</td>
<td>3.19</td>
<td>2.75</td>
</tr>
<tr>
<td>0.1 ppm</td>
<td>2.56</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>Basa in crab</td>
<td></td>
</tr>
<tr>
<td>1000 ppm</td>
<td>3.61</td>
<td>1.31</td>
</tr>
<tr>
<td>100 ppm</td>
<td>6.46</td>
<td>1.35</td>
</tr>
<tr>
<td>10 ppm</td>
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</tr>
<tr>
<td>1 ppm</td>
<td>4.33</td>
<td>1.43</td>
</tr>
<tr>
<td>0.1 ppm</td>
<td>4.33</td>
<td>1.08</td>
</tr>
</tbody>
</table>

* Each sample was analyzed three times a day, and the assay was repeated on two different days. 
* The pooled standard deviation (sp) was calculated using equation 1. The pooled mean (Xp) was calculated by substituting individual means for variances in equation 1. The intra-assay and inter-assay CVs were calculated using equation 2 (Grotjan and Keel 1996).

**Equation 1**

\[ s_p = \sqrt{\left(\sum_{i=1}^{n} (df_i \times s_i^2)\right) / \sum_{i=1}^{n} df_i} \]

Where:
- \( s_i \): individual standard deviation (SD)
- \( df_i \): degrees of freedom (n-1, n: sample size of individual experiment)

**Equation 2**

\[ CV (%) = (sp / Xp) \times 100 \]
Table 4 The false-positive and false-negative rates of the sandwich ELISA

<table>
<thead>
<tr>
<th>Result of Index</th>
<th>Result of the sandwich ELISA (%)</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

a. The false-positive and false-negative rates of the sandwich ELISA were calculated based on a total of 1704 individual samples. These samples were tested by the sandwich ELISA on different days in different experiments. All 1380 positive samples tested positive. All 324 negative samples tested negative.
Figure 1. Immunoreactivity of MAb 8F5 by indirect ELISA with cooked samples from fish, shellfish, land animals and food additives. The samples were coated 2 μg/well in the plate. The MAb 8F5 supernatant was diluted 1:5 in PBST with 1% BSA. Results were expressed as the mean of A415 ± SEM.
Figure 2. SDS-PAGE (A) and immunoblot (B) analyses of the antigenic protein of MAb 8F5 in cooked fish samples. The amounts of samples loaded on the 12% SDS-PAGE are 15 μl per lane for cooked fish samples.
Figure 3. SDS-PAGE analysis of the protein profile of raw fish samples (A) and cooked fish samples (B). The amounts of samples loaded on the 12% SDS-PAGE were 6 μl per lane for raw fish samples and 20 μl per lane for cooked fish samples.
Figure 4. Comparison of SDS-PAGE analysis of salmon tropomyosin and cooked fish samples in the absence (A) and presence (B) of urea in the gel. The amounts of samples loaded on the 12% SDS-PAGE are 10 μl per lane for cooked fish samples and 2 μg per lane for salmon tropomyosin. The concentration of urea in gel is 6M.
Figure 5. Comparison of immunoblot analysis of salmon tropomyosin and cooked fish samples using MAb 8F5 in the absence (A) and presence (B) of urea in the gel. The amounts of samples loaded on the 12% SDS-PAGE are 10 μl per lane for cooked fish samples and 2 μg per lane for salmon tropomyosin. The concentration of urea in gel is 6M.
Figure 6. SDS-PAGE (A) and immunoblot (B) analyses using MAb 8F5 with purified salmon 36 kDa protein and purified salmon tropomyosin. The amounts of samples loaded on the 12% SDS-PAGE are 2 μg per lane for purified salmon 36 kDa protein and 6 μg for purified salmon tropomyosin.
Figure 7. Comparison of the amino acid composition profiles of the purified Atlantic salmon 36 kDa protein and purified Atlantic salmon tropomyosin. The reference amino acid composition of Atlantic salmon tropomyosin was reported by Vejborg and others (2008).
Figure 8. The protein sequence of the tryptic digested fragment of the 36 kDa protein. The sequence of the tryptic digested fragment is circled.
Figure 9. Determination of the amino acid sequence of the MAb 8F5 epitope using a peptide array. The amino acid sequence of MAb 8F5 epitope is circled. The fish-specific regions are underlined.

MDAIKKKMQM LKLDKENALD RAEGAEKD KK AAEKSKQLE DOLVALOKKL KGTEDELDKY SESLKDAQE K LEVAEKTATD AEAADVASLN R IQLVEEEDL RAQERLATAL TKLEEAKEA A DESERGMKVI ENRASKDEEK MELQDIQLKE AKHIAEADR KYEEVARKLV IIESDLRTE ERAELSEGKC SELEEELKTV TNLKSLA A EKYSQ KEDK YEEIKVLT D KLKEAETRAE F AERSVAKLE KTIDDEDEL Y AQLKYKAI SEELDNALND MTSI
Figure 10. Immunoreactivity of PAb B by indirect ELISA with raw and cooked samples from fish, shellfish, land animals and food additives. The samples were coated 2 μg/well in the plate. The PAb B (6 mg/ml) was diluted 1:3,000 in PBST with 1% BSA. Results are expressed as the mean of A415 ±SEM.
Figure 11. Immunoreactivity of PAb BD by indirect ELISA with cooked samples from fish, shellfish, land animals and food additives. The samples were coated 2 $\mu$g/well in the plate. The PAb B (1 mg/ml) was diluted 1:1,000 in PBST with 1% BSA. Results are expressed as the mean of A415 ±SEM.
Fig. 12. SDS-PAGE (A) and immunoblot analyses (B) of cooked fish samples and fish tropomyosin using PAb BD. The amounts of samples loaded on the 12% SDS-PAGE are 6 μl per lane for cooked fish samples and 2 μg per lane for purified salmon tropomyosin. STD: standard
Figure 13. Schematic representation of sandwich ELISA.
Figure 14. Selection of the antibodies for the development of the sandwich ELISA. The experimental condition for both capture antibody and detection antibody is 1:10,000 for PAb B (6 mg/ml); 1:3,000 for PAb BD (1 mg/ml); 1:10 for MAb 8F5 (supernatant) in PBST with 1%BSA. Results are expressed as the mean of A415 ±SEM.
Figure 15. Optimization of raw (A) and cooked (B) sample dilutions of the sandwich ELISA. The capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. The detection Ab was PAb BD (1mg/ml) diluted 1:1,000 in PBST with 1%BSA. The optimized dilution factor for both raw and cooked fish samples was 1:2. Results were expressed as the mean of A415 ±SEM.
Figure 16. Immunoreactivity of the sandwich ELISA with raw samples from fish, shellfish, land animals and food additives. All the samples were diluted 1:2 in PBS. The abbreviations of the samples were listed in Appendix C. In PBST with 1%BSA. The detection Ab was PAb BD (1mg/ml). Capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. The experiment was repeated twice in triplicate. Results were expressed as the mean of A415 ±SEM. The absorbance at 415 nm was plotted against the concentration of the samples.
Figure 17. Immunoreactivity of the sandwich ELISA with cooked samples from fish, shellfish, land animals and food additives. Results were expressed as the mean ± SEM. The experiment was repeated twice in triplicate.

The capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. All the samples were diluted 1:2 in PBS. The detection Ab was PAb BD (1mg/ml) diluted 1:1,000 in PBST with 1%BSA. The abbreviations of the samples were listed in Appendix C. The absorbance was expressed as the mean of A415 ±SEM. The experiment was repeated twice in triplicate.
Figure 18. Immunoreactivity of the sandwich ELISA with salted fish samples. The capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. The detection Ab was PAb BD (1mg/ml) diluted 1:1,000 in PBST with 1%BSA. The optimized dilution factor for both raw and cooked fish samples was 1:2. Results were expressed as the mean of A415 ±SEM, P<0.05. The experiment was repeated twice in triplicate.
Figure 19. Immunoreactivity of the sandwich ELISA with smoked fish samples. The capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. The detection Ab was PAb BD (1mg/ml) diluted 1:1,000 in PBST with 1%BSA. The optimized dilution factor for both raw and cooked fish samples was 1:2. Results were expressed as the mean of A415 ±SEM, P<0.05. The experiment was repeated twice in triplicate.
Figure 20. Immunoreactivity of the sandwich ELISA with canned fish samples and fishmeal. The capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. The detection Ab was PAb BD (1mg/ml) diluted 1:1,000 in PBST with 1%BSA. The optimized dilution factor for both raw and cooked fish samples was 1:2. Results were expressed as the mean of A415 ±SEM, P<0.05. The experiment was repeated twice in triplicate.
Figure 21. Limit of detection (LOD) of fish (pollock, whiting, basa) in crab using the sandwich ELISA. The raw (A) and cooked (B) spiked samples were prepared in five adulteration levels (1000 ppm, 100 ppm, 10 ppm, 1 ppm, 10.1 ppm) by v/v. The capture Ab was PAb B (6mg/ml) diluted 1:1,000 in PBST with 1%BSA. The detection Ab was PAb BD (1mg/ml) diluted 1:1,000 in PBST with 1%BSA. The optimized dilution factor for both raw and cooked fish samples was 1:2. Results are expressed as the mean of A415 ±SEM, P<0.05. The experiment was repeated twice in triplicate.
## APPENDIX C

Sample list

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## Abbreviation List

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<td>Aldehyde Phosphate Dehydrogenase</td>
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Table of commercial kits for the detection of food allergens.
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Table of commercial kits for the detection of food allergens (continued)
<p>| Table of commercial kits for the detection of food allergies (continued) |
|---|---|---|---|---|---|
| <strong>Food</strong> | <strong>Type</strong> | <strong>Tech.</strong> | <strong>Incubation</strong> | <strong>Result</strong> | <strong>Bioagent</strong> |
| Cow's milk | Standard ELISA | 40 min | 0.25 p.p.m. | Radiosensitive Cat allergen | Milk |
| Sheep's milk, goats' milk | Standard ELISA | 40 min | 0.5 p.p.m. | Radiosensitive Cat allergen | Milk |
| | Latex-enhanced ELISA | 10 min | 1 p.p.m. | Latex from Breddle <em>tularensis</em> | Beef |
| | Real-time PCR | unknown | 10 p.p.m. | Serotonin@ALLERGEN Avoided | Avoided |
| | Arrhythmia device | 1 min | | Latex from Allium asparagus | Avoided |
| | Standard ELISA | 40 min | 2.5 p.p.m. | Radiosensitive Cat allergen | Avoided |
| | ATP-assisted device | 30 seconds | 0.1-2.5 p.p.m. | Allergene | Soybean |
| | ATP-assisted device | 30 seconds | 0.1-2.5 p.p.m. | Allergene | Soybean |
| | ATP-assisted device | 30 seconds | 0.1-2.5 p.p.m. | Allergene | Soybean |
| | ATP-assisted device | 30 seconds | 0.1-2.5 p.p.m. | Allergene | Soybean |
| | Latex-enhanced ELISA | 5 min | 5 p.p.m. | G. trichia (Crambe) | Wheal |
| | | | | G. trichia (Crambe) | Wheal |
| | Sheep's milk | Standard ELISA | 2.5 % | Wheal, Pseudo ELISA KU | Wheal |
| | | | | Pseudo ELISA KU | Wheal |
| | | | | Radiosensitive Cat allergen | Windmill |</p>
<table>
<thead>
<tr>
<th>Test</th>
<th>Method</th>
<th>Sample Volume</th>
<th>Detection Time</th>
<th>Detection Limit</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Celiac</td>
<td>Western blot</td>
<td>Serum, Tissue</td>
<td>10 min</td>
<td>20 mg/ml</td>
<td>Genetests</td>
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<tr>
<td>R-Biopharm</td>
<td>Enzyme-linked immunosorbent assay</td>
<td>Serum</td>
<td>10 min</td>
<td>5 mg/ml</td>
<td>R-Biopharm</td>
</tr>
<tr>
<td>RIDASCREEN® AST</td>
<td>Immunochromatographic assay</td>
<td>Serum</td>
<td>10 min</td>
<td>2 mg/ml</td>
<td>R-Biopharm</td>
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<tr>
<td>R-Biopharm</td>
<td>Sandwich ELISA</td>
<td>Serum</td>
<td>10 min</td>
<td>5 mg/ml</td>
<td>R-Biopharm</td>
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<tr>
<td>R-Biopharm</td>
<td>Sandwich ELISA</td>
<td>Serum</td>
<td>10 min</td>
<td>2 mg/ml</td>
<td>R-Biopharm</td>
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<tr>
<td>Genetests</td>
<td>Enzyme-linked immunosorbent assay</td>
<td>Serum</td>
<td>10 min</td>
<td>5 mg/ml</td>
<td>Genetests</td>
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Table of commercial kits for the detection of food allergies (continued)
<table>
<thead>
<tr>
<th>Family Name</th>
<th>Product Name</th>
<th>Application</th>
<th>Type</th>
<th>Sensitivity</th>
<th>Cross Reactivity</th>
<th>No. Sensitivity</th>
<th>Sample Type</th>
<th>Detection Method</th>
<th>Response Time</th>
<th>Confirmation Method</th>
<th>RUO</th>
<th>CE Mark</th>
<th>FDA</th>
<th>Price</th>
<th>Detection Limit</th>
<th>Storage</th>
<th>Shelf Life</th>
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<tbody>
<tr>
<td>Allergen</td>
<td>Detection Method</td>
<td>Detection Equipment</td>
<td>Detection Time</td>
<td>Detection Limit</td>
<td>Detection Result</td>
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<td>Cow, Sheep, Goat</td>
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<td>90 min</td>
<td>0.5 ppm</td>
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<td>Positive</td>
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Table of Commercial Kits for the Detection of Food Allergens (continued)
REFERENCES


Chen L, Lucas JS, Hourihane JO, Lindemann J, Taylor SL & Goodman RE. 2006. Evaluation of IgE binding to proteins of hardy (Actinidia arguta), gold (Actinidia chinensis) and green (Actinidia deliciosa) kiwifruits and processed hardy kiwifruit concentrate, using sera of individuals with food allergies to green kiwifruit. Food & Chemical Toxicology 44(7):1100-1107.


FDA 2012: http://www.fda.gov/Food/FoodSafety/ProductSpecificInformation/Seafood/RegulatoryFishEncyclopediaRFE/ucm064456.htm


Mikita CP & Padlan EA. 2007. Why is there a greater incidence of allergy to the tropomyosin of certain animals than to that of others? Medical Hypotheses 69(5):1070-1073.


Shanti KN. 1993. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. Journal of Immunology 151(10):5354-5363.


BIOGRAPHICAL SKETCH

Education:

2007-2012 PhD, Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, FL

2005-2007 PhD student, Department of Biology, Georgia State University, Atlanta, GA

2001-2004 Masters, Department of Animal Science, Tunghai University, Taichung, Taiwan

1997-2001 Bachelors, Department of Animal Science, Tunghai University, Taichung, Taiwan

Awards:

2012 Scholarship awarded by the Betty M. Watts Memorial Found for Food Science, Florida State University

2012 University Award, Florida State University

2012 2nd place in the Graduate Student Research Paper Competition, 2012 Annual Meeting of Institute of Food Technologists (IFT)

2011 Scholarship awarded by the Florida-China Linkage Institute, Florida-China Linkage Institute

2010 1st place in the J. Mac Geopfert Developing Scientist Award, 2010 Annual Meeting of International Association of Food Protection (IAFP)

Research Experience:

2007-2012 Research assistant, Florida State University

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2006 Mentor in the Biotech Scholars program, Georgia State University

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2009-2010 Teaching assistant in the Food Science Lab, Florida State University
**Journal Articles:**


**Poster Presentations:**


