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Pooling of Monoclonal Antibodies for Rapid Detection of Commercially Important Fin Fish

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POOLING OF MONOCLONAL ANTIBODIES FOR RAPID DETECTION OF COMMERCIALLY IMPORTANT FIN FISH

By

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TABLE OF CONTENTS

List of Tables vii

List of Figures viii

Abstract ix

INTRODUCTION 1

1.1 Background 1

1.2 Rationale and Significance 3

1.3 Hypotheses, Objectives and Experimental Approaches 6

LITERATURE REVIEW 9

2.1 Fish Allergy 9

2.2 Fish Allergen 11

2.3 Relevant Guidance and Regulations Related to Fish Allergy 13

2.4 Fish Detection Methods 15

2.5 Effect of Storage on Fish Muscle Protein 28

2.6 Effect of Processing on Fish Muscle Protein 28

MATERIALS AND METHODS 30

3.1 Materials 30

3.2 Methods 31

3.2.1 Preparation of Seafood Protein Extracts 31

3.2.2 Preparation of Other Protein Extracts: Meat Protein Extracts 32

3.2.3 Preparation of Other Protein Extracts: Nonmeat Protein Extracts 32

3.2.4 Preparation of Selected Fish Protein Extracts Stored for Different Lengths of Time . 32

3.2.5 Preparation of Protein Extracts from Representative Fish with Different Processing Methods 33

3.2.6 SDS–Polyacrylamide Gel Electrophoresis and Western Blot 33

3.2.7 Indirect Non-Competitive Enzyme-Linked Immunosorbent Assay (iELISA) 34

3.2.8 Dot Blot 35

3.2.9 Assay Validation 36

3.2.10 Statistical Analysis 36

RESULTS AND DISCUSSION 37

4.1 Selectivity of Three MAbs Using iELISA and Dot Blot 37

4.1.1 MAb 8F5 Could Recognize 57 of 63 Commercially Important Fish Samples 37

4.1.2 MAb 2G9 and MAb 2F3 Could Fill the Gap with Minor Cross Reactivity With Other Fish Species 39

4.1.3 All Three MAbs Had No Cross Reactivity with Non Fish Samples 40

4.2 Characterization of the Antigenic Proteins of MAbs 8F5, 2G9 and 2F3 41

4.2.1 SDS-PAGE Showed Different Patterns Among Selected Fish Samples 41

4.2.2 Western Blot Demonstrated Different Antigenic Proteins Identified by Three MAbs 42

4.3 Detection of All 29 Selected Common Food Fish Species Using Pooled MAbs 43

4.4 Effects of Storage Length, Cooking Time and Processing on the Immunoreactivity of Selected Fish Using iELISA and Dot Blot 44

4.4.1 Effect of Fish Sample Storage on Immmunoreactivity 44

4.4.2 Effect of Fish Extract Storage on Immmunoreactivity 45

4.5 Effects of Processing on the Immunoreactivity of Selected Fish Using the Developed Assay 46

4.5.1 Heating 46

4.5.2 Salting and Smoking 47

4.6 Validation of the iELISA and Dot Blot Assays 48

CONCLUSIONS 50

APPENDICES 51

A: TABLES 51

B: FIGURES 72

C: ABBREVIATION LIST 85

D: BUDGET 87

REFERENCES 88

BIOGRAPHICAL SKETCH 107

LIST OF TABLES

1 Identified fish allergens....13

2 Comparisons of the cost of different immunoassays (Xu et al., 1989)....26

3 Comparison of western blot, dot blot and ELISA....27

4 Processed fish sample information....51

5 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 against fish species (cooked) using iELISA and dot blot....52

6 Immunoreactivity of individual and pooled MAbs 8F5, 2G9, 2F3 against fish species (raw) using iELISA and dot blot....57

7 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 and three pooled MAbs against selected fish (cooked) using indirect ELISA and dot blot....62

8 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 and three pooled MAbs against selected fish (raw) using indirect ELISA and dot blot....65

9 Intra-assay variability of iELISA....68

10 Inter-assay variability of iELISA....69

11 Sensitivity of assays....70

12 Specificity of assays....70

13 Overall accuracy of assays....71

LIST OF FIGURES

1 Schematic representation of indirect non-competitive ELISA (iELISA) 72

2 Schematic representation of dot blot 72

3 Immunoreactivity of MAb 2G9, 8F5 and 2F3 to non-fish samples using iELISA(A) and dot blot(B) 73

4 SDS-PAGE protein profile of extracts from 9 species of cooked fish 74

5 Antigenic protein banding patterns of cooked fish extracts determined by western blot using MAb 8F5(supernatant) 75

6 Antigenic protein banding patterns of cooked fish extracts determined by western blot using MAb 2G9 (supernatant) 76

7 Antigenic protein banding patterns of cooked fish extracts determined by western blot using MAb 2F3 (supernatant) 77

8 Immunoreactivity of individual MAbs 2G9, 8F5 and 2F3 pooled to non-fish samples using iELISA (A) and dot blot (B) 78

9 Immunoreactivity of MAb 2G9 against yellow fin tuna meat and extract of different storage (meat storage and extract storage) length using dot-blot and indirect ELISA 79

10 Immunoreactivity of MAb 8F5 against amberjack meat and extract of different storage length using dot-blot (A) and iELISA (B) 80

11 Immunoreactivity of MAb 2F3 against swordfsih meat and extract of different storage length using dot-blot (A) and iELISA (B) 81

12 Immunoreactivity of MAbs 2G9, 8F5 and 2F3 against yellow fin tuna, amberjack and swordfish of different cooking length using dot-blot 82

13 Immunoreactivity of MAbs 2G9 (1:3), 8F5(1:20) and 2F3 (1:3) against yellow fin tuna (A), amberjack (B) and swordfish (C) of different cooking length using iELISA 83

14 Immunoreactivity of MAbs (8F5, 2F3, 2G9 and pooling of three) to raw and cooked processed fish using iELISA(A) and dot blot (B). 84

ABSTRACT

Due to its nutritional and health benefits, the consumption of fish as an important dietary protein source is increasing. However, these benefits are not universal; fish is also one of the eight major allergenic foods or food types and is responsible for 0.4% of food-hypersensitive patients in the U.S. Approximately 1.1 million Americans suffer from various symptoms of fish allergy, and this number is increasing. At present, food manufacturers must rely on good manufacturing practice (GMP) and hazards analysis and critical control point (HACCP) plans to eliminate the risk of undeclared allergens in their products. Although immunoassays using antibodies are widely accepted by regulatory agencies as a rapid and sensitive method for screening and monitoring substances in food and agricultural products, the vast species genetic variation of fish has so far failed to identify a single protein that would be suitable for use as an antigen marker for the detection of all species of fish in an immunoassay.

Three anti-fin fish MAbs (8F5, 2G9 and 2F3) were previously developed in our laboratory against cooked crude fish protein extract from red snapper, yellow fin tuna and swordfish, with characteristic antigenic proteins at 36kD, 25kD, 30-150kD, respectively. The selectivity of these individual MAbs to 63 commercially important fish species has been examined using indirect enzyme-linked immunosorbent assay (iELISA) and dot blot; none showed an ideal pattern of recognition of the target single species without some level of cross reactivity or cross reaction with some or all of the other fish species tested. However, the cross reactivity pattern of each MAb complements the other two and none cross react with non-fish samples, which could be utilized to recognize a complete spectrum of most commercially important fish species. The pooled MAbs(1:1:1) could thus be used as a standard reagent for the detection of common fish species, including under various processing conditions. Dot blot immunoassay, which requires less highly trained personnel, showed an equivalent performance in detecting the presence of fish. Both iELISA and dot blot assay yield high sensitivity, specificity and productivity. These assays could provide a user-friendly tool not only for the regulatory agencies charged with enforcing food laws but also to help ensure the safety of fish sensitive patients.

CHAPTER ONE

INTRODUCTION

1.1 Background

Fish plays an important role in the human diet, providing a valuable source of high quality proteins, beneficial polyunsaturated fatty acids, and lipid-soluble vitamins. Due to the increased awareness of these nutritional benefits, the consumption of fish as a protein source is rising. In 2010, the contribution of fish to global diets reached a record amount of approximately 17 kg per person per year on average (FAO 2011), supplying over three billion people with at least 15 percent of their average animal protein intake.

However, the benefits of eating fish are not always received by everyone. Four percent of the total population in the United States (US) (Sicherer et al. 2004) suffers from some type of food allergic disorder. Fish are not always 'innocuous' or 'harmless', whether consumed or via inhalation. Fish allergy is one of the eight major allergenic foods or food types that are responsible for more than 90% of the immunoglobulin E (IgE)-mediated food allergies in humans (Bahna 2004; FDA 2005; Kobayashi et al. 2006; Sicherer and Sampson 2006).

Fish allergies are a potentially serious emerging public health problem in developed countries. The prevalence of fish allergies in the United States is thought to be 0.4% of the adult population, with approximately 1.1 million Americans suffering from various symptoms of fish allergy, and this number is increasing (Sampson 1999; Sicherer et al. 2004). Clinical symptoms include cutaneous reactions (urticaria, angioedema, eczema), respiratory symptoms (asthma, rhinitis), gastrointestinal symptoms (vomiting, diarrhea, cramps) and systemic reactions (cardiovascular symptoms, even fetal anaphylactic shock) (O'Neil and Helbling 1993; Swoboda et al. 2002; Van Do, Hordvik et al. 2005). These symptoms, which are similar to those of other IgE-mediated food allergies, are often induced very rapidly, within seconds to an hour of exposure.

Fish allergens are usually proteins or glycoproteins that are resistant to heat and protease with a molecular mass from 10 to 70 kDa (Griesmeier 2009). Although only a small portion of the proteins in fish are allergenic, the allergens that cause Ig-E mediated responses belong to a number of different protein families (Besler 2001). Parvalbumin is a major fish allergen that causes more than 50% cases of fish allergies, so previous fish detecting tools have generally focused on anti-parvalbumin (Chen et al. 2006; Gajewaki and Hsieh 2009) probing. However, as there are numerous cases where patients are allergic to non-pavalbumin proteins, the most practical way of protecting consumers is to avoid any fish ingredients that could potentially cause an allergic reaction. Since 2005, the Food Allergen Labeling and Consumer Protection Act (FALCPA) (FDA 2005) has required manufacturers to declare on the label any allergenic foods or ingredients derived from allergenic foods that may be present and food manufacturers have depended on good manufacturing practices and hazards analysis and critical control point plans (HACCP) to avoid the presence of contaminating allergens in their products. This is clearly not a satisfactory situation and there is therefore an urgent need for a rapid test capable of detecting all common fish species that are potential sources of allergens in food products, which is the only way to protect fish allergic consumers (Vierk et al. 2002).

Immunoassays are widely accepted by regulatory agencies as a rapid and sensitive method for screening and monitoring substances in food and agricultural products (Rao and Hsieh 2008). The usefulness of enzyme-linked immunosorbent assays (ELISA) and dot blots for the quick detection and semi-quantification of allergens or target proteins of potentially allergenic food have been clearly established (Blais and Phillippe 2000). This study therefore sought to develop an effective and rapid immunoassay for the detection of commercially important fish. Several previously developed monoclonal antibodies (MAbs), 8F5, 2G9 and 2F3, were used in this study. Preliminary experiments revealed that MAb 8F5 (IgG 2a), which recognizes a 36kD protein, reacted strongly with the great majority of the 63 commercially important finfish species tested, failing only to react with tuna species (yellow fin tuna, blue fin tuna and albacore), escolar, wahoo and swordfish. MAb 2G9 (IgG1) recognizes the major antigenic 25kD protein, reacted with tuna, escolar and wahoo, while MAb 2F3 (IgG1) reacted with swordfish and weakly cross reacted with several other fish species. In order to achieve the comprehensive detectability

needed for commercially important fish, the three MAbs 8F5, 2G9 and 2F3 will be pooled and used simultaneously. The test developed as a result of this study is expected to not only discourage the illegal practice of mislabeling fish ingredients at the retail and restaurant levels, but may be a life saver for those unfortunate individuals who suffer from a serious fish allergy.

1.2 Rationale and Significance

Although several studies (Hsieh et al., 2009; Hsieh et al. 2009; Asensio et al. 2003, 2008, 2009; Faeste and Plassen 2007) have been done on the identification of important fish species using ELISA, there are currently no anti-fish protein antibody-based ELISA available for the detection of all commercially important fish. For the protection of fish sensitive consumers and for the enforcement of food labeling laws, an effective, rapid and consumer friendly analytical method for detecting fish protein in food for human consumption is urgently needed. The significance of this research is derived as follows:

1. The importance of developing an all-fish assay

Fish allergy is a major public health concern for food-hypersensitive patients who are sensitive to fish, who make up 0.4% of the population in the United States. Approximately 1.1 million Americans suffer from various symptoms of fish allergy, and this number is increasing (Sampson 1999; Sicherer et al. 2004). The importance of developing an all-fish assay is threefold. Firstly, this will help protect fish allergic people and their families. Vulnerable consumers rely solely on the information provided by the label or menu, but this is not necessarily 100% accurate. As a result, they may be exposed to hidden allergens that might be harmful to them. Secondly, the government must continue to bear the ever-increasing cost of providing medical care for fish allergy incidences due to accidental encounters with undeclared allergens (http://www.foxnews.com/health/2011/05/06/food-allergies-cost-500-million-year-study-finds/). A reliable and rapid method for the detection of the presence of fish protein is therefore critical to successful efforts to enforce food labeling laws. Thirdly, effective detection methods could help reduce food recalls for manufacturers. The food industry suffers from huge losses each time

there is a recall because of undeclared fish allergens in their products (http://www.fda.gov/Safety/Recalls/ucm241863.htm). Since 2010, there has been an increasing number of recalls by governments worldwide due to undeclared fish ingredients in non-fish products (http://www.fda.gov/Safety/Recalls/ucm258317.htm). At present, there is no commercially available user-friendly test method capable of detecting commercially important food fish proteins. Thus, there is a great need for food manufacturers to have access to a sensitive, affordable solution for their allergen prevention plans.

2. The importance of developing anti-fish protein antibody-based indirect non-competitive ELISA (iELISA) and dot blot tests for fish protein detection

Immunochemical techniques (Hsieh et al., 1995; Hsieh, 2009; Asensio et al. 2003, 2008, 2009; Faeste and Plassen 2007), have been extensively utilized in protein detection. ELISA (Enzyme-Linked ImmunoSorbent Assay) is one of the most popular immunoassay formats. ELISA uses enzymes as an amplifier to detect the presence of antibody-antigen binding in an aqueous solution on a solid polymer plate (Engvall et al. 1971). A modified form of ELISA, dot blot (Hawdes et al., 1982; Herbrink et al., 1982), uses nitrocellulose and DBM paper for the detection of antibodies. Instead of using an ELISA plate, the nitrocellulose membrane is used as the test matrix, a simplified version of the western blot method (Towbin et al. 1979; Renart et al. 1979; Burnette 1981).

Both ELISA and dot blot were chosen in this study for the detection of commercially important fish due to their multiple advantages. First, both are based on using antibodies to bind antigens (proteins), and are thus appropriate for signaling the presence of fish protein. Secondly, ELISA and dot blot offer a large-scale screening and field test capability and are simple, rapid, sensitive, reliable and low-cost techniques (Crowther, 2000). Thirdly, both methods are readily adapted for commercial test kits and results can be obtained in few hours.

The dot blot technique (Hawdes et al., 1982) requires even less equipment than does ELISA. No plates, ELISA washer or ELISA reader are needed, as samples are simply spotted onto a coated membrane. The dot blot then provides a “yes” or “no” answer to the presence of target antigen

simply by visual inspection, making them very suitable for use in the field. Also, dot blots have a much longer shelf life for coated samples and results in the membrane than ELISA plates (Herbrink et al., 1982). However, their relatively high cost and high numbers of false positives/negatives may be a problem for higher protein absorbing nitrocellulose membranes in dot blot assays. The indirect non-competitive ELISA(iELISA), the simple form of ELISA that is closest to dot blots, will also be performed as a predictor to verify the dot blot results. Thus, both an iELISA and dot blot assay will be performed and optimized using pooled MAb for fish protein detection.

3. Using pooled anti-fish antibodies for the detection of fish protein from common fish species

Monoclonal antibodies (MAb) are favored as the standard reagents for immunodetection due to their unlimited supply at low cost once successfully developed. One obstacle is the cross reactivity of a single MAb, which has long been the reason for the failure to develop an effective species-specific antibody (Faeste and Plassen 2007). Although this unexpected cross reactivity could be utilized to probe a group of fish species (Hsieh et al. 2009), the "desired cross reactive" is not sufficient to cover all fish species within a group. Since no single MAb has yet been found that is capable of recognizing all the commercially important fish without any cross activity, it should be possible to pool a group of antibodies with different species-specificity to encompass a comprehensive coverage of common fish species. Thus, pooled MAbs may serve as a standard reagent for the development of a user-friendly immunoassay as a rapid, field screening method.

The three monoclonal antibodies (MAbs) which were used in this study are MAbs 8F5, 2G9, and 2F3 raised against crude heat-treated protein extract from red snapper, yellowfin tuna and swordfish, respectively. From preliminary experiments, MAb 8F5, which belongs to the IgG 2a subclass, recognizes an antigenic, thermally stable protein of 36kD revealed by western blot. It can be used to indicate the presence of a wide range of commercially important fish species but not tuna, escolar, wahoo or swordfish. However, the MAbs 2G9 and 2F3, both of which belong to the IgG1 subclass, can recognize those fish species that MAb 8F5 fails to identify: MAb 2G9 recognizes a 25 kD antigenic protein in tuna, escolar and wahoo, while MAb 2F3 recognizes multiple bands (30-150 kD) in swordfish and weakly cross reacts with several other fish species.

As a result, in order to achieve a comprehensive detectability for most commercially important fish, pooling MAbs 8F5, 2G9 and 2F3 should facilitate developing an affordable and rapid immunoassay based on dot blot, enabling fish allergic consumers, restaurants and manufacturers to test whether fish ingredients are present in food regardless of heat treatment of the samples.

4. The importance of studying the effects of storage, cooking, and processing on the detectability of the target fish proteins

A test that is only capable of detecting fresh fish will not always reflect the detectability of product consumers/agencies/manufacturers will require. To validate this assay, several aspects needed to be considered. Firstly, the fish products that consumers are able to access are distributed to grocery stores through a cold chain, which means storage for times ranging from days to months. The consumer may also keep fish in their freezer for some time before using it. Secondly, fish products in the marketplace are not always frozen and are often processed through methods such as smoking or salting. Thirdly, with more and more consumers preferring to dine out or purchase ready-to eat packs due to time constraints, the cooking time of the fish also needs to be considered. Inspection agencies and manufacturers often retain fish samples or extracts for a period of time for food safety documentation purpose. Such differences in storage length, cooking time and processing methods are likely to have significant effects on the food protein structure (Tejada 2001; Careche et al. 2002; Saeed and Howell 2002) and consequently the antigenic epitopes, which might increase, decrease, or remain unchanged in terms of the immunoreactivity and detectability of the target fish proteins (Sathe and Sharma 2009; Sletten et al. 2010). Thus, it is necessary to examine the effects of storage, cooking time and processing on the immunoreactivity of several representative samples of fish products.

1.3 Hypotheses, Objectives and Experimental Approaches

MAb 8F5 (IgG 2a) raised against thermal-stable crude protein extract of red snapper was previously developed in our lab. It recognizes a 36kD protein and reacts strongly with all 63 commercially important finfish species tested except for tuna species (yellow fin tuna, blue fin

tuna and albacore), escolar, wahoo, mahi-mahi and swordfish. The MAb 2F3 (IgG1), which was raised against cooked crude extract of swordfish, reacts with swordfish and weakly cross reacts with several other fish species. The third MAb, 2G9 (IgG1), was raised against thermal-stable crude protein extract of yellowfin tuna and cross-reacts not only with tuna species (yellowfin tuna, blue fin tuna, albacore), but also the non-tuna fish escolar, wahoo and mahi-mahi. By pooling the above three MAbs, their complementary species-specificity should enable them to cover all the species of commercially important finfish of interest.

Thus, the central hypothesis guiding this research was:

A rapid user-friendly immunoassay detection method for the detection of all commercially important fish species can be developed by using pooled MAb 8F5, MAb 2G9, and MAb 2F3.

The specific objectives were to:

a) verify the cross reactivity and molecular weights of the target antigenic proteins of MAbs 8F5, 2G9 and 2F3;

b) develop an indirect non-competitive ELISA (iELISA) and a user friendly dot blot for the detection of fish proteins using pooled MAbs; and

c) study the effect of storage and processing on the immunoreactivity of target fish proteins.

The experimental approaches were as follows:

Objective One: verify the cross reactivity and molecular weights of the target antigenic proteins of MAbs 8F5, 2G9, 2F3. For this purpose, fish and non-fish protein samples were extracted by 0.15M NaCl, and the protein extracts used to determine their immunoreactivity using individual MAbs 8F3, 2G9, and 2F3 in iELISA, western blot and dot blot. Neutral salt solutions of low ionic strength (0.15 M NaCl) were used to extract more sarcoplasmic proteins, which is suited to distinguishing fish species (http://www.fao.org/fishery/topic/14869/en). iELISA and dot blot were also be used to verify the cross reactivity of fish, non-fish meat and food protein additives.

In order to verify the molecular weights of the target antigenic proteins, SDS-PAGE and western blot were performed.

Objective Two: develop an indirect non-competitive ELISA (iELISA) and a user friendly dot blot for the detection of fish protein using pooled MAbs. For this purpose, the fish samples selected were subjected to extraction with 0.15M NaCl. The samples were undergo immunoreactivity assessment by pooling 3 MAbs (8F5 2G9, and 2F3) and using iELISA and dot blot.

Objective Three: study the effect of storage and processing on the immunoreactivity of target fish proteins. For this purpose, the fish samples selected were subjected to freezing storage, cooking, and processing. The samples selected were undergo immunoreactivity assessment by individual MAb as well as pooled MAbs using iELISA and dot blot.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fish Allergy

Food allergens are food component(s) that induce the production of IgE antibodies, causing mediator to be released from mast cells and basophils and resulting in immediate hypersensitive reactions (Aalberse, 2000; Sicherer and Sampson 2006). Food allergens contained in the diet enter the interior of the body through the alimentary tract (Lopata et al. 2009). Together with milk, wheat, soy, shell fish, tree nuts, peanuts, and eggs, fish is one of "big eight" major allergen foods (Bousquet et al. 1998; FAO 1995). Symptoms of fish allergy, which are similar to those of other IgE mediated food allergies, usually appear immediately after exposure (minutes to an hour). Clinical symptoms of fish allergy include urticaria, angioedema, asthma, rhinitis, vomiting, diarrhea and anaphylaxis (Rosmilah et al. 2005). The prevalence of allergy to particular foods varies geographically and is related to regional dietary practices and the extent of exposure (Hourihane 1998). The incidence of food allergies has estimated at anywhere from 2.3% to 30.4% of the population (Faeste and Plassen 2008), although systematic and statistically validated date on true food allergies are globally lacking (Tanabe 2008). Fish allergy often appears at an early age, and tends to be persistent, in contrast to some other food allergies with similar young ages of onset that are typically resolved at school age as tolerance develops (Eigenmann and Sicherer 1998; Peng and Shyur 2001). In fish allergic patients, the ingestion of fish, inhalation of the vapors generated during cooking, and skin contact may all lead to a variety of IgE-mediated symptoms. Fish hypersensitivity is frequently encountered in coastal countries, especially where considerable numbers of the inhabitants work in fish-related industries and fish consumption is high (Van Do, Elsayed et al. 2005; Kobayashi et al., 2006).

Fish allergy is a global public health concern and is one of the major food causing allergies affecting adults in the US. The recently increases in the level of production and consumption of seafood have led to more frequent reports of allergic reactions in both occupational and domestic settings (O'Neil and Helbling 1993; Sicherer et al. 2004). Symptoms of fish allergy usually appear immediately (minutes to an hour) and can range from urticaria and dermatitis to angiedema, diarrhoea, and asthma, in some cases, even fatal anaphylaxis. Approximately 1.1 million Americans suffer from fish allergy, a reported 0.4% of the population (estimated 2.3% to 30.4% of the population), and this number is increasing (Sampson 1999; Munoz-Furlong et al. 2004). According to an investigation conducted in 1997 by the Japanese Ministry of Health and Welfare, approximately 20% of food allergic adults in Japan are sensitive to fish (Hamada et al. 1997) and fish allergy affects approximately 0.5% of the general population in the United Kingdom (Emmett et al., 1999). Among the 54 episodes of food-induced anaphylaxis in children recorded in one study, 30% were caused by fish (Novembre et al. 1998), and 0.4% to 0.5% of children in the general population have been diagnosed as allergic to codfish (de Martino and Peruzzi 1993) in Italy. Crespo and Pascual (1995) reported that in a group of 355 children with food allergy in Spain, 30% had a fish allergy. Another study (Boyano and Esteban 1987) reported that fish was the third most common food allergen after eggs and cow's milk in Spain. Another study estimated that the frequency of fish allergic individuals in Norway was approximately 0.1% of the population (Aas 1987) and around 0.3% of the adult population in Sweden was also fish-allergic (Bjornsson and Janson 1996). Studies from Finland have estimated that 3% of the country's 3-year olds were fish-allergic (Saarinen and Kajosaari 1980).

Generally, fish allergic patients are allergic to most types of fin fish. Due to the high allergic cross-reactivity among different types of fish, fish sensitive patients who are allergic to one type of fish are also likely to have (or to develop) allergies to others and are therefore advised to avoid consuming all fish (Hansen and Bindslev-Jensen 1997). However, patients who are allergic to only a few species are particularly vulnerable to a life-threatening risk of consuming mislabeled or substitute fish in an unknown situation. A case has been reported of a patient who had never been allergenic to any fish except canned tuna developing symptoms of angioedema of the lips, hands, and penis/scrota by eating salmon (Asero 1998). More such cases could have occurred without the cause becoming apparent.

Since there is no standard test for diagnosis, reports of fish allergy depend heavily on self-reported data. Thus, the real prevalence of fish allergy remains unknown. From the educational nutrition and health aspect, fish is considered as a food with high quality proteins, polyunsaturated fatty acids and lipid-soluble vitamins. As global fish consumption has increased by 35% in the past 30 years (Delgado and Wada 2003), there may be a great risk to those susceptible individuals. Every sector of the global food chain has been involved (aquacultures, processing, etc) in incidents involving the contamination of allergic ingredients and it is ever more likely that large populations could be affected by food allergy. Contamination by fish allergen ingredients could occur during shipping and storage, processing or from carry-over due to inadequate cleaning of shared processing equipment (Huggett and Hischenhuber 1998; Van Hengel, 2007). Visual observation is not always a reliable way to determine whether allergen cross-contact is actually occurring. The contamination can be intentional or unintentional and there are many ways for allergens to be hidden in food, for example through misleading labels, allergenic foods that can contaminate other safe foods, carelessness, food that is listed by an uncommon term, and ingredient switching, among others (Anibarro and Seoane 2007).

2.2 Fish Allergen

The major allergen in finfish is a low-molecular-weight protein termed parvalbumin (Aas, 1966; Eva et al. 2006; Syed et al. 2010), and this protein has been responsible for more than 50% of the fish allergic cases recognized by IgE. Parvalbumins are a family of calcium-binding proteins that play an important role in muscle relaxation (Heizmann and Berchtold1982; Muntener and Kaser 1995). Parvalbumin is the most studied fish allergen, originally discovered in the form of the Gad c 1 from codfish (*Gadus callarias*). Gad c 1 (originally designated as allergen M) constitutes approximately 0.05 - 0.1 % of the white cod muscle tissue. It has a low molecular weight of approximately 10-13 kDa and acidic pI values (pI 5.0), and is water soluble and resistant to heat treatment as well as enzymatic degradation (Elsayed and Aas 1971; Aas and Elsayed 1975). Several studies (Rosmilah et al. 2005; Das-Dores and Chopin 2002) have suggested that bands at 24, 38 and 51kDa may be parvalbumin (Gad c1) dimers, trimers and tetramers, respectively, in

G. morhua's (a species of Atlantic cod called Gadus morhua) Atlantic cod research recognized by anti-parvabumin IgE.

The strength of the immune response is highly dependent on the foreignness of one species to another. Parvalbumins are highly expressed in the muscles of lower vertebrates, such as fish and frogs, and are present in lesser amounts in higher vertebrate animals such as humans (Gerday 1982). Species of fish such as cod, salmon, Alaska pollock, mackerel, thornback ray (Thatcher and Pechere 1977; Lindstrom et al. 1996; Swoboda et al. 2002; Hamada and Tanaka. 2003; Van Do, Elsayed et al. 2005) have two phylogenic linkeage types of parvalbumins, α and β, and these have different isoelectric points. The β-parvalbumins have a relatively lower pI (5.0) and more acidic amino acids than α -parvalbumin, the major cause of fish allergy (Haiech et al. 1973; Goodman et al. 1979). Within an individual species, parvalbumins exist in different types of fish muscles: white muscle generally contains more parvalbumin than dark muscle, which makes the dark muscle tissue of fish much less allergenic than the white muscle tissue (Rehbein and Kindiger 1984; Kobayashi et al. 2006). Hence, dark muscle fish such as tuna and swordfish, which contain less white muscle, are considered less allergenic foods for parvalbumin-sensitive patients.

Other than parvalbumin, several additional fish allergens have also been reported (Table 1). A 41 kDa heat labile protein, aldehyde phosphate dehydrogenase (APDH), has been reported as a major allergen in codfish, golden snapper and red snapper (Das-Dores and Chopin 2002). A 51 kDa protein has been found in both red snapper and golden snapper (Rosmilah et al. 2005), and a 46kDa protein in golden snapper that act as allergens. Additionally, patients allergic to a 25kDa protein in swordfish (Kelso, et al., 1996), 41kDa -46kDa proteins in tuna, and 18kDa and 45kDa proteins in tilapia have been reported. However, none of these were well characterized (James et al. 1997). Fish collagen (100 kDa) has also been identified as a cross reactive fish allergen (Sakaguchi and Hori 1999). Another study (Kuehn et al. 2009) demonstrated that two bigeye tuna proteins of 120 and 240 kDa, corresponding to the α-chain and β-chain (dimer of the α-chain) of collagen (probably type I collagen), reacted with sera from fish allergic patients.

Table 1 Identified fish allergens

Name	Molecular Weight	Found in species	References
Parvalbumin	10-13 kDa	cod, salmon, Alaska pollock, mackerel, car, thornback ray, carp, salmon etc	Elsayed and Aas 1971; Aas and Elsayed 1975; Untersmayr et al. 2006
-	25kDa	Swordfish	Kelso et al.1996
APDH	41 kDa	codfish, golden snapper and red snapper	Das-Dores and Chopin 2002
-	41kDa -46kDa	Tuna	James 1997
-	45kDa	Tilapia	Ebo et al. 2010
-	51 kDa	red snapper	Rosmilah et al. 2005
-	51 kDa	golden snapper	Rosmilah et al. 2005
Fish collagen	100 kDa	cod, albacore, yellow fin tuna, salmon, saurel, eel, sea bream and mackerel.	Sakaguchi and Hori 1999; Hamda et al. 2001; Yata et al. 2001; Kondo et al. 2006
α-chain collagen	120 kDa	bigeye tuna	Kuehn et al. 2009
β-chain collagen	240 kDa	bigeye tuna	Kuehn et al. 2009

2.3 Relevant Guidance and Regulations Related to Fish Allergy

For allergic consumers it is particularly important to obtain full information about potential allergens contained in a food product from clear and accurate labeling because the presence of undeclared allergens as contaminants in food products may pose a major risk for sensitized persons (Arjon and van Hengel 2007; Poms et al. 2004). In the United States, legislation has

been enacted to provide a high level of health protection for allergic consumers (Arjon and van Hengel 2007). The US Congress's the Food Allergen Labeling & Consumer Protection Act (FALCPA) became law on January 1, 2006 and many of the provisions of this law will benefit food allergic consumers by requiring clearer labeling of the ingredients in allergenic food and listing the ingredients in descending order of predominance. FALCPA recognizes milk, eggs, fish, shellfish (shrimp, prawns, crab and lobster), peanuts, soy, wheat, and tree nuts as the most common allergenic foods and requires the use of 'plain English language' to identify the sources of ingredients derived from these commonly allergenic sources (FDA, 2004). The presence of common allergenic foods or ingredients derived from such foods can be provided in one or more ways: (1) The ingredient can simply be identified on the ingredient list, for example fish, peanuts and wheat starch; (2) The source of the ingredient can be identified parenthetically in the ingredient list, for example natural flavor (milk), lecithin (soy), casein (milk); and/or (3) A 'contains' statement can be used immediately below the ingredient statement, for example 'Contains: fish, shellfish, and soybean' (FDA, 2004). FALCPA also requires the designation of the species on labels for fish and crustacean shellfish, although there might be confounding issues with certain ingredients. For example, fish gelatin is primarily composed of proteins derived from fish and can be made from the skins of multiple fish species such as cod, flounder or haddock (Hansen et al. 2004). The identification of fish gelatin could lead to serious restrictions in the diets of allergic consumers and create potential confusion.

In Europe, Directive 2000/13/EC, as amended by Directive 2003/89/EC, sets out a list of food allergenic ingredients, including fish, which must always be identified when used in the manufacture of pre-packaged foods (OJ L 308, 25.11.2003 p.15). This applies no matter how small the amount used and includes its use as part of a compound ingredient. However, some highly processed ingredients derived from fish, including fish gelatin used as a formulation aid (carrier) in vitamin and carotenoid preparations, and isinglass used as a fining agent in wine, listed in Directive 2003/89/EC are unlikely to trigger reactions in sensitive individuals and are therefore exempt from the requirement to label with reference to that source food. These exceptions were made based on an evaluation by the European Food Safety Authority (EFSA) of the evidence currently available (http://www.efsa.europa.eu/). The European Food Safety Authority (EFSA) web site also provides information on food allergen labeling in Europe. The

scientific panel responsible for food allergies has, for example, provided a number of opinions on the scientific basis for the labeling legislation and exemptions from it. This will allow time for further scientific data to be assessed by EFSA as it is published. Derived ingredients not present on this list are considered to be capable of eliciting adverse reactions and should be handled accordingly (Humieres and Wal 2004)

Both FALCPA and the Labeling Directive only cover pre-packaged food, so foods sold loose or from catering outlets are not covered by current allergen labeling rules. As many of the most serious allergic reactions occur in restaurants and other food-service establishments where full label disclosure of ingredients is typically not practiced (Sampson et al. 1992; Yunginger et al. 1988), this is clearly a problem and there is a strong case to be made for the labeling of potential allergic food in the above locations.

The British and Australian governments both provide guidelines and recommend the use of an allergen risk assessment tool to harmonize the application of allergen precautionary labeling. The recommended approach for limiting the possibility of allergen contamination is through a HACCP (Hazard Analysis and Critical Control Point) program. The guide lists key areas that food companies need to consider to control allergen risk, including employee training and supervision, raw material sourcing and storage, production scheduling, equipment and premises design, manufacturing (e.g. cleaning procedures, control of rework), labeling, and post-manufacturing controls. The guide introduces Voluntary Incidental Trace Allergen Labeling (VITAL), which is a risk-based methodology that food producers can use to apply appropriate allergen precautionary labeling (http://www.allergenbureau.net/). In the U.S., the Hospitality Institute of Technology and Management have produced guidance for caterers and retailers to help them avoid specific allergens (Snyder, 2005).

2.4 Fish Detection Methods

Since there is no cure for food allergies, successful detection is a potential life-saver for fish allergic patients. There are several methods used for the detection of allergen, but most focus on

diagnosis rather than prevention. Human Ig-E can be used in a double blind placebo-controlled food challenge (DBPCFC) for establishing thresholds and diagnosis of patients, but in addition to the high risk this poses to highly sensitive patients it is very time-consuming (Sampson et al. 2001). Also, the human Ig-E based radioallergosorbent test (RAST) and enzyme allergosorbent test (EAST) may be restricted in availability due to the limited supply of human serum (Poms et al. 2004), thus making it difficult to standardize for routine food analysis. Using indirect methods to detect the presence of fish is thus a more attractive option. There are various methods for detecting fish ingredients in order to prevent allergy, but these fall into two main categories. In the first, the detection targets the allergen (protein) itself, which is effective if the food only contains this particular allergen. However, "fish" is a vast family with a number of allergenic proteins and significant species diversity, so these methods cannot be efficiently used for a screening test that works for every fish species. The second approach is to develop a marker that indicates the presence of the offending food. These markers could be used either to identify individual fish species that cause allergenic reactions, or to recognize the finfish family in general in order to provide information to fish sensitive patients.

Morphology

In the past few years, there has been a great deal of time and effort spent on developing ways to identify fish species. The US Food and Drug Administration (FDA 2011) provides free species information data on common market fish via the database Regulatory Fish Encyclopedia (RFE), which helps federal, state, and local officials and purchasers of seafood identify species substitution and economic deception in the marketplace. The RFE covers a number of commercially relevant fish species for sale in the U.S. and provides high-resolution photographs in the form of scanned digital images (jpg format) of both the whole fish and their common product forms (including fillets, steaks, or whole crustaceans) that may be used for visual comparison. However, as a result of market globalization, large numbers of different species and inadequately trained people employed in species identification make this approach of limited utility. Even when it does work, after processing at a centralized food plant the product has often lost its morphological characteristics (Yancy et al 2008), thus it is no longer possible to identify the species.

Chromatography

Chromatography, a set of laboratory techniques for the separation of mixtures, has been applied to identify species based on an examination of protein profiles of fish samples (Carrera et al. 2010; Wang et al. 2010). Separation of sarcoplasmic protein by high performance liquid chromatography (Armstrong et al. 1992) has successfully been applied to detect 15 commonly editable fish in Australia. Kuuutinen and coworkers (1998) used reversed-phase high-performance liquid chromatography (RP-HPLC) to separate sarcoplasmic fish protein for species identification. Chromatographic methods can be capable of differentiating individual fish species, but they are less effective in detecting adulterated species in mixtures or cooked fish because of the increased complexity of the chromatographic patterns. In addition, the requirements of expensive instruments, highly trained staff and laborious sample preparation procedures are the major draw backs of these technologies.

Near infrared microscopy

In near infrared (NIR) microscopy the infrared beam is focused using a dedicated microscope on each particle of sample on a sample holder and the near infrared spectrum is recorded. The identification of fish meat patterns is made using spectral features measured in the near infrared region (1100-2500 nm) of the electromagnetic spectrum (Abbas et al. 2010). Recent studies (Shi et al. 2009; Cozzolino et al. 2010) have applied NIR for the identification of soybean adulteration in fish meal. The advantage is that it allows the user to obtain spatial and spectral information simultaneously to characterize the sample. However, like most spectroscopic technology, there is a risk due to the bias of the mathematics of modeling which may affect the accuracy of the results (an acceptable level of confidence is a 95% rate of detection of animal particles at a level of 0.1%). Also, the expensive and non-portable equipment and the complexity of sample preparation prevent its wider or routine application. Moreover, NIR can only discriminate the higher taxonomic groups of species.

Electrophoretic techniques

Electrophoretic techniques can be used for fish species identification because fish products are composed of muscles made from proteins. Most electrophoretic methods use sarcoplasmic

proteins for species identification in raw meat because heat denatures and insolubilizes most native sarcoplasmic proteins, and protein extracts of heat-processed meat result in only a few faint bands (Hsieh 2005). Analysis of fish samples by electrophoretic methods must be made by comparing the patterns obtained to those of authentic samples run simultaneously. The major methods discussed below perform protein profile characterization by separating proteins based on either their pI value, or their molecular size.

Isoelectric focusing. Isoelectric focusing (Righetti 1977) is the official method for identifying fish species used by the FDA. The principle of isoelectric focusing is based on separating different molecules by differences in their isoelectric point (pI), which is the point at which the protein has an overall net charge of zero. Differences of only a few hundredths of a pH-unit in isoelectric points are sufficient to resolve proteins from each other. In many applications, closely related proteins have to be separated. Examples are the differentiation of protein isoforms or enantiomeres. Such problems have been successfully solved by isoelectric focusing (IEF). In contrast to other electrophoretic techniques, pH is not kept constant throughout the whole system. Instead, the sample components migrate electrophoretically through a stationary pH-gradient. Proteins will migrate until they reach the pH point in the gradient at which the charge of the protein equals zero (pH = pI). The protein is said to focus at this point. This focusing also results in a concentration of individual proteins (Lundstrom, 1981). One of the advantages of IEF is that it has a lower detection limit since it has a wider sample zone. Another advantage of this technology is that separation in IEF does not require denaturation of the proteins, so any kind of subsequent investigation, such as activity staining (e.g. to find separated enzymes) or antibody detection, is not precluded. As the protein profiles of the 77 most common commercialized fish species are already listed in the FDA database, comparing a fish sample with a reference should identify the species through patterns. However, because all these methods need the sarcoplasmic protein to be in its native state, it is not always practical for species identification of cooked or highly processed fish. Instead, sodium dodecylsulphate polyacrylamide gel electrophoresis (Scobbie et al. 1988) or urea-isoelectric focusing (An et al. 1988) can be used to analyze denatured protein solubilized in sodium dodecylsulphate and urea, respectively.

SDS-PAGE . The principle of SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide GEl) is based on the separation of proteins with different molecular weight through an electrical field. SDS-PAGE uses an anionic detergent (SDS) to denature proteins so the protein molecules become linearized. One SDS molecule binds to two amino acids, so the charge to mass ratio of all the denatured protein in the mixture becomes constant. These protein molecules move in the gel (towards the anode) on the basis of their molecular weights only and are separated accordingly (Laemm li 1970).

According to a large collaborative study of 10 commercially important species (Etienne and Marc 2000), IEF is better for parvalbumin (which has acid protein bands) characterized species such as gadoid, while SDS-PAGE is more suitable for tuna and salmon species, which are characterized by their neutral proteins. Another successful study of species substitution of smoked eels compared results from SDS-PAGE and Urea IEF to identify the smoked gravid salmonids, as the water-soluble protein alters due to the effect of the processing methods used (Sotelo et al. 1992). However, these methods are limited by the complicated sample preparation and extraction of cooked samples required, which makes electrophoretic methods difficult for the routine analysis of cooked meat samples (Hsieh et al. 1995).

Two-dimensional gel electrophoresis (2-DE). Since IEF separates proteins according to their isoelectric point and SDS-PAGE separates proteins with different molecular weights, a two dimensional map of electrophoresis, namely 2-DE, can be constructed (O'Farrell et al. 1975; Lopez 2007). The authenticity of fish and fish products can be analyzed by looking at the spots of positions of protein determined by their pI and molecular weight. The application of computer science technology to segment, align and match the 2-DE images of protein profiles have allowed the investigation of spots and made quantitative analysis possible. By separating myofibrillar proteins using 2-DE, Martinez and Friis (2004) investigated the freshness and quality deterioration of raw fish under various storage conditions. However, it is very hard to apply this approach to small and high molecular weight proteins, preventing its wider application for fish protein studies.

All of these electrophoretic techniques can only identify individual species of fish, which makes the technique helpful to fish allergic patients who are sensitive to only one fish species, but of limited use for the general allergic patients who need to avoid all fish species. These methods are time consuming and need professional lab techniques, thus making them unsuitable as a quick screening tool for detecting the presence of fish ingredients.

DNA-Based assays

DNA techniques have become very important and are widely used nowadays for fish detection. Most existing immunological methods that detect native proteins as the characterizing species component have limited application for differentiating species in highly processed animal products because heat and processing are likely to denature the target protein, leading to subsequent loss of analytical specificity (Hsieh et al. 2005). DNA based methods can resolve this problem because of the following advantages: a) DNA is a relatively stable molecule, allowing the analysis of both processed and heat treated food products (Unseld et al. 1995; Beneke and Hagen 1998); b) DNA carries an organism's genetic information, and the information content of DNA is greater than protein due to the degeneracy of the genetic code as one goes from DNA to protein (Wolf et al. 2000); and c) DNA is a remarkably stable molecule allowing its extraction from all kinds of tissue due to the ubiquity of DNA in every type of cell (Wolf et al. 2000; Wolf and Luthy 2001).

Most early DNA based methods were based on polymerase chain reaction (PCR) and hybridization to specific probes. Probes prepared from genomic DNA or cloned DNA were hybridized with target DNA and detected by color development or autoradiography. These methods are specific and useful for the identification of fish species, but insufficient for the identification of closely related species or quantification of adulteration levels. Amplification of species-specific targets by PCR has proved to be a sensitive and powerful technique, with two major advantages over protein analysis: samples heated to as high as 120°C can still be analyzed and discrimination between related species is possible (Bellorini et al. 2005; Hsieh et al. 2005).

PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism). The basic technique for detecting RFLPs involves fragmenting a sample of DNA using a restriction

enzyme that can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot (Southern 1975) procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments, which are complementary to the probe (Humayun et al. 1977). Compared to other DNA-based methods for species identification, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of mt-DNA has offered the most widely used DNA technology for species identification (Bellagamba et al. 2001). The advantages of PCR-RFLP are many: a) one universal PCR-primer system in combination with a few restriction enzymes (RE) can be sufficient for species identification (Meyer et al. 1995); b) no references are necessary once the restriction patterns of the species of interest have been determined; and c) a careful selection of RE prevents ambiguous results caused by intraspecies polymorphisms (Wolf et al. 1999). According to a study by Mackie (1999), four segments of cytochrome b that could function as species identification were isolated from DNA from raw and canned tuna.

DNA barcoding. This must be followed by a secondary technique for species identification. DNA barcoding, a technology that amplifies and sequences the mitochondrial cytochrome oxidase I (COI) gene (approximately 650 bp from the 5′ region), has been widely applied in various biological fields and has a proven ability to differentiate closely related species in studies ranging from forensic sciences, through molecular systematics (Hsieh, 2003) to fish product identification (Civera 2003; Quinteiro et al. 1998). Extensive DNA barcode libraries have been developed, most notably the Barcode of Life Data Systems (BOLD). COI-sequenced species information, including the origin and current locations of voucher specimens (Cooper et al. 1998), has been provided, greatly expanding the utility of this technology. Information on over 10,000 species of fish has been stored in the BOLD database. These COI barcodes are gathered from several sources including the Fish Barcode of Life Initiative (FISH-BOL) (Wilson 1997; Scholz 1998) and the Marine Barcode of Life Initiative (MarBOL,http://www.marinebarcoding.org). However, a common problem for all these methods is that different nucleotide patterns shown within the same species can make these methods

unreliable. Thus it is necessary for genebank records to be verified before use (Rasmussen and Morrissey 2008).

Although DNA sequencing methods generate the highest information content for DNA-based species identification and the FDA is considering incorporating DNA barcodes into the Regulatory Fish Encyclopedia to help with fish species identification, the major drawback of this technology is it is very time consuming and costly, and is specific to certain species of fish rather than fish species as a whole (Rasmussen et al. 2009).

Immunoassays

Immunoassays are biochemical tests that measure the concentration of a substance in a biological liquid using the specific binding reaction of an antibody or antibodies to its antigen. Both the presence of antigen or antibodies can be measured. Immunological methods are suitable for assaying various analytes in complex mixtures with minimal requirement of sample preparation.

Specific human serum IgE is necessary for the characterization of fish allergen, but it is not suitable for reliable fish detection in food products since the specificity of IgE from sensitized individuals differs considerably and the amount of serum is usually limited. Additionally, multiple sensitivities and/or cross-reactivities to more than one allergenic fish are often present in human serum IgE. All those properties of sera obtained from individual patients prevent standardization and commercialization of methods based on human IgE (Besler 2001). In order to overcome the disadvantages associated with the use of human serum IgE, immunoassays relying on IgG antibodies raised in animals such as rabbits, mice, goats, sheep or chickens have been developed and are widely used. Antibodies may be either monoclonal or polyclonal. Polyclonal antibodies are often available and usually have high affinity for the antigen, but they also contain nonspecific antibodies against unknown antigens that may be present in a crude mixture. For this reason, many polyclonal antibodies are affinity purified to yield specific antibodies.

Well characterized monoclonal antibodies (MAbs) are low in cost, available in unlimited amounts, and are of consistent specificity and affinity to the immunoreagent. They are ideally used for species identification (Huang et al. 1995; Sheu and Hsieh. 1998; Zade et al. 2001; Kim et al. 2004; Gajewski and Hsieh 2009). However, the initial cost of developing cell lines for monoclonal antibody production is high. The development of monoclonal antibodies for the detection of various species in animal products is very difficult, but once developed, those MAbs can be applied in various immunoassays for maximum success (Djurdievic 2005). The major challenge for immunoassays is the difficulty of developing specific antibodies with specificity to a single species or all fish species without cross reactivity. There are thousands of species of marine fish and fresh water fish, so the development of species-specific monoclonal antibodies would appear to be impossible. However, pooling a group of monoclonal antibodies with different cross reactivities within the fish species could provide comprehensive coverage of all fish species and serve as a standard reagent to develop a user-friendly assay for a quick, field screening method.

Enzyme-linked immunosorbent assay (ELISA). ELISA, or Enzyme-Linked ImmunoSorbent Assay, has become one of the most popular immunochemical formats. ELISA uses enzymes as an amplifier to detect antibody-antigen binding in the aqueous solution in a solid polymer plate by Dr Perlmann's research group (1971). Before the development of ELISA, the only option for conducting an immunoassay was radioimmunoassay, a technique that relies on radioactively-labeled antigens or antibodies. In radioimmunoassay, the radioactivity provides the signal that indicates whether a specific antigen or antibody is present in the sample. Because radioactivity poses a potential health threat, a safer alternative was sought. ELISA substitutes a non-radioactive signal for the radioactive signal originally used. Indirect non-competitive ELISA (iELISA) is a simple form of ELISA. Here, the antigen is coated into the well of the polymer plate for absorption, and a blocking step used to maintain the absorption and prevent non-specific protein binding properties. Adding the first antibody will the result in strong binding with the coated antigen. The signal has to be associated with the presence of the target molecular, which is why the enzyme has to be linked to an appropriate second antibody (Morgancapner et al. 1979). When enzymes react with appropriate substrates, this will cause a change in color, which is serves as the indicator. Usually this colored product is soluble and the change in color is

recorded by a spectrometer. ELISA has found extensive applications in animal protein identification because it is both simple and specific. It does not require large and expensive scientific equipment and only uses small quantities of immunoreagents. The performance of ELISA depends on the quality of the antibody.

Generally, work related to fish species identification is scarce, partly due to the wide variety of fish species that are commercialized. Nevertheless, in recent years several polyclonal antibodies and monoclonal antibodies have been produced against fish muscle proteins, for example to discriminate raw and cooked grouper among other less valued fish species (Asensio et al., 2003) and to identify pangasius catfish species (Hsieh et al. 2009). Among the different types of immunoassay used for detection, only the ELISA technique and the lateral flow "dipstick" technology have gained commercial acceptance for fish species identification and limited numbers of commercial ELISA and lateral flow test kits have been developed. The EZ Pangasius assay, by ELISA Technologies Inc. is an easy-to-use field test that will quickly identify cooked or raw fillets of pangasius (http://www.elisa-tek.com/elisa-technologies-news/ez-pangasius-rapid-test-kit).

Immunoblot. Immunoblot tests indicate the presence of an antigen using the specificity of antigen-antibody interactions in a membrane. The protein is blotted onto the membrane by different means, and the binding of antibodies with the particular target antigens is then measured. There are two types of blots: the western blot and the dot blot.

Western blot is used to detect the presence and molecular weight of antigens in a crude mixture and to compare immunological cross-reactivity among proteins. It combines two techniques during blotting: polyacrylamide gel electrophoresis (PAGE) and transfer. The separated proteins are then subjected to an immunoassay to detect the presence of proteins. Using this combination of techniques makes it possible to identify target proteins and confirm their identity by molecular mass based on their position relative to standard proteins of known molecular weight (Towbin et al. 1979). The use of western blot for fish identification is rare, but it supports the characterization of antigenic proteins (Rahman 2007; Hsieh et al. 2009). The western blot is the only method capable of detecting both the molecular weight and presence of antigens, but the

procedure is relatively complex, so it requires highly trained personnel working in a laboratory setting. Additionally, the western blot is costlier compared to the dot blot and ELISA assays because of the reagents and instruments used for the SDS-PAGE and transfer process. Another limitation of the western blot method is that it might not be suited to the analysis of native antigens since the addition of SDS would destroy the conformational epitopes. However, linear epitopes would not be affected.

A dot blot represents a simplified version of the western blot method and is an alternative form of ELISA. Samples containing antigen are dotted on a membrane, probed with labeled antibody, and then color developed. No separation on PAGE or transfer process is needed, so the dot blots only provide information about the presence of the antigen, not its molecular weight. Estimation of antigen concentration is possible if known standards are included on the blots, although the blots do not provide the accuracy of other immunoassays such as sandwich ELISA or competitive ELISA because of the difficulty of accurately measuring the color density. However, the ease of manipulating the membranes and the ability to test many samples simultaneously have made dot blot a popular technique for rapid screening. Dot blot is used to quickly determine the optimal concentration of antigen and labeled antibody to be used in a western blot. The enzyme-substrate used in dot blot is different from those used in ELISA because the intent is to form an insoluble colored product that stays on the membrane. The color intensity and width of the protein band together indicate the concentration of the target protein in the sample extract.

The dot blot technique (Table 2) offers significant savings in time, money and experimental expertise, as gel electrophoresis and complex blotting procedures for the gel are not required. It has a high potential for use as a user-friendly commercial product to generate a straightforward “yes” or “no” answer for screening, though it offers no information on the size of the target antigen. Furthermore, if target antigens of different sizes are both reacted with the antibody, they will still appear as a single dot. Dot blots therefore can only confirm the presence or absence of a biomolecule that can be detected by the antibody.

While the ELISA /western blot test has been used for a number of research and commercial purposes, it has limitations that make it unsuitable for use in the field or in a consumer’s home

where no equipment is available (Table 3). In such situations, a simple, inexpensive colorimetric assay without the need for instruments could have many field diagnostic applications. Also, only small amounts of antigens are needed in dot blot to achieve sufficient sensitivity to ELISA. A dot blot requires extremely small amounts of antigen per test and allows for reprobes several times for different antibodies. The number of applications of dot blot for a fish adulteration detection is limited. A Spanish research group (Dominguez et al. 1997) developed a dot blot assay using polyclonal antibodies for detecting species substitution of halibut for sole.

Table 2 Comparisons of the cost of different immuneoassays (Xu et al. 1989)

Method	Quantity of sample required per test (mL)		Time required	Cost per test (USD)
	Serum	Supernatant		
Dot blot	0.01-0.02	0.02	3-4 h	0.008
Western blot	0.03	3.00	6-7 h	22.00
Non-commercial ELISA	0.001	0.10	3-4 h	0.32
Commercial ELISA	0.003	0.12	3-4 h	3.00

Table 3 Comparison of western blot, dot blot and ELISA

	Western blot	Dot blot	ELISA
Purpose	Presence of antigenic protein of MAb	"Yes" or "No" screening for the presence of antigen	Quantitative/ Qualitative measure of present of antigen or antibody
Material	Nitrocellulose membrane, other cloth	Nitrocellulose membrane, other cloth	Use ELISA plate
Preliminary steps	SDS-PAGE needed first (Electrophoresis apparatus, technique), Transfer unit; Power supply; Page is versatile; poisons, need level 2 or 3 bio lab training	Draw the dot for showing the result	-
Qualitative	Yes	Yes	Yes
Quantitative	Semi	Semi	Could be Quantitative
Color development	Insoluble color developed	Insoluble color developed	Soluble color developed
Spectrophotometer	No	No	Optional
Separation of antigen	Yes (SDS-PAGE)	No	No
Shelf life of results	Storage for month	Storage for month	Several days
Results preserved	Long term	Long term	Few days
Samples per test	Limited sample loaded	many samples screened simultaneously	many samples screened simultaneously
Lab personnel	Highly trained	User-friendly	Need some training
Cost	High	Low	Medium
Not applicable	Conformation epitope detecting	Molecular weight of antigenic protein	Molecular weight of antigenic protein

2.5 Effect of Storage on Fish Muscle Protein

Fish are usually stored in frozen conditions (- 20^{o}C or lower). However, there may be changes in the muscles that affect the protein profile in fish meat during cold storage. Firstly, the formation of ice crystals causes cryo-concentration of solutes, partial dehydration and dislocation of water in the muscle, all of which accelerate protein denaturation and aggregation. Secondly, fatty acids (FA) have been reported to cause acceleration of protein denaturation and aggregation in gadoid species, and therefore the conditions favor protein aggregation (Tejada 2001; Careche et al. 2002; Saeed and Howell 2002). FAs bind mainly with myofibrillar proteins, causing rapid denaturation and formation of protein-protein bonds among neighbor proteins, which also accelerates aggregation. The types of protein-protein bonds formed include secondary interactions (ionic and hydrogen bonds, hydrophobic interactions) and covalent bonds (disulfide and non-disulfide covalent bonds). The rate of formation and the percentage of protein aggregated by the different types of bond depend on the species, the temperature and the length of time in frozen storage. Longer frozen storage and destruction of the structure of fish muscle increase the formation of covalent bonds (Sletten et al. 2010). Protein aggregation leads to decrease in protein solubility, extractability and Ca2+-ATPase activity of myosin and actomyosin and also to changes in protein hydrophobicity (Rodriguez-Casado et al. 2007; Rawdkuen 2010). These changes may have different effects on food protein structure and the antigenic epitopes, which might increase, decrease, or have no effect on the immunoreactivity and detectability of the target fish proteins (Sathe and Sharma 2009; Sletten et al. 2010).

2.6 Effect of Processing on Fish Muscle Protein

Smoking and salting are preservation techniques that have been used since ancient times. According to Rora and coworkers (1998), approximately 40% to 50% of Atlantic salmon production reaches the market as a cold smoked product. The process of smoking includes salting, drying and then the smoking itself, which may be conducted at cold or hot temperature. As a previous study showed (Sletten et al. 2010), heating leads to the denaturation of fish

proteins owing to the rupture of their secondary and higher structures. As a consequence of denaturation, protein may aggregate and form new bonds. The drying processing involved in the smoking may enhance oxidation and rancidity and cause a slight reduction in protein quality.

In contrast, the hot smoking process is known to cause a significant ($P < 0.05$) decrease in protein from smoked mackerel muscle as compared with that from nonsmoked fish muscle (Bhuiyan et al. 1986). Another study investigated chemical changes in pike perch, rainbow trout and eel after hot smoking (Kottelat 1997); some differences in water, protein, lipid, ash and carbohydrate contents of the fish were found to be significant ($P < 0.01$). Using polyacrylamide gel electrophoretic analysis, most of the bands disappeared after smoking and storage owing to denaturation of the fish proteins.

Processing such as salting and smoking is likely to affect the capacity of allergenic foods to trigger allergic reactions by modulating the integrity of the allergic proteins (Paschke 2002). Since the epitopes on allergens, which bind to IgEs of fish sensitive individuals, can be either linear stretches of amino acids, or conformational structures where the amino acids are not sequential but are in each other's vicinity as a result of the protein's three-dimensional structure. Processing that affects the binding of IgEs to the epitopes in an allergic protein either by changing the three-dimensional conformation or by cleaving the linear epitopes. Thus, it would not be surprising if processing reduced the allergenic capacity (Monaci 2006). However, an increase in the allergenic capacity as a result of food processing is also possible; processing could even reveal hidden epitopes that were previously buried within the three-dimensional structure of the allergen and protein or peptide modification resulting from food processing might lead to the formation of novel structures with allergenic capacities, which might result in increased IgE binding.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Acetic acid, bovine serum albumin (BSA), citric acid, egg albumin, glycerol, hydrogen chloride (HCl), 2-mercaptoethanol (2-ME), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium phosphate monobasic anhydrous (NaH_2PO_4), sodium dodecyl sulfate (SDS), Tween 20, Whatman no. 4 filter paper, 96-well polyvinyl microplate (Costar 2595) and 96-well polystyrene microplate (Costar 9018) were purchased from Fisher Scientific (Fair Lawn, NJ). Alkaline phosphatase (AP) conjugate substrate kit, dye reagent concentrate, gelatin, goat anti-mouse IgG (H+L) AP (anti-IgG-AP) conjugate, Mini-Protean III electrophoresis cell, Mini Trans-Blot Electrophoretic Transfer Cell, nitrocellulose membrane, protein assay kit II, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl buffer (pH 8.8), 30% acrylamide/bis solution, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), 20 mM Tris-buffered saline (TBS, pH 7.5), Tris-glycine buffer, Tris-glycine-SDS buffer and Precision Plus Protein Kaleidoscope standards for western blotting were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ammonium persulfate, EZBlue Gel Staining Reagent, hydrogen peroxide (H_2O_2) solution (30%, g/g), goat anti-mouse IgG (Fc-region specific) horseradish peroxidase (anti-IgG–HRP) conjugate and Ponceau S were purchased from Sigma-Aldrich Co. (St. Louis, MO). Methanol was purchased from VWR International (West Chester, PA). Absolute ethanol was purchased from Pharmco Products Inc. (Brookfield, CT). All chemicals and reagents were analytical grade. All solutions were prepared using distilled, deionized, and sterilized pure water from the NANOpure Diamond ultrapure water system (Barnstead International, Inc., Dubuque, IA).

Soy protein concentrate was purchased from Central Soya Co. (Fort Wayne, Ind.). Whey protein concentrate was purchased from Davisco Foods International, Inc. (Eden Prairie, MN). Fish, shellfish, beef, pork, chicken breast, chicken thigh, lamp, rabbit, turkey breast, turkey thigh, nonfat dry milk (NFDM) and CRISCO pure canola oil were purchased from local supermarkets (Tallahassee, FL). Deer and horse meat were obtained from a private source.

MAbs. The MAbs 8F5, 2G9, 2F3 used in this study were previously developed against crude thermal-stable protein extract from cooked (100°C, 15 minutes) red snapper, yellow fin tuna, and swordfish, respectively, by Dr.Hsieh (unpublished data).

3.2 Methods

3.2.1 Preparation of Seafood Protein Extracts

Seafood samples were stored at -20°C prior to sample preparation. The product information for these seafood samples is shown in Table 4. All the seafood products were cut into small pieces. For raw samples, about 8 grams of each sample were weighed in a beaker covered with aluminum foil and 9 fold (w/v, 1 gram raw sample /9 mL extract buffer) saline (0.15M sodium chloride) added to the mashed sample to extract the soluble proteins. For the cooked samples, about 8 grams of each sample were weighed in a beaker covered with aluminum foil which was then sealed with autoclave tape and heated in a boiling water bath for 8 min at 100°C. After cooling to room temperature, each cooked sample was mashed into fine particles. Then 2 fold (w/v, 1 gram cooked sample/ 2 mL extract buffer) of the extraction buffer (0.15 M sodium chloride) was added to the mashed samples to extract the soluble thermostable proteins. All raw and cooked samples were held overnight at 4°C. The mixtures were then centrifuged at 3,220 × g for 30 min at 4°C, using the Eppendorf 5810R centrifuge (Brinkmann Instruments, Inc., Westbury, NY). The supernatants were filtered through Whatman no. 4 filter paper, dispensed into 1.5 ml microcentrifuge tubes (Fisher) and stored at -20°C until use. The protein concentration of each extract was determined using the Protein Assay Kit II (Bio-Rad). BSA was used as the protein standard.

3.2.2 Preparation of Other Protein Extracts: Meat Protein Extracts

Fat and connective tissues were trimmed off meat samples (pork, beef, chicken, turkey, and lamp). The lean meat samples were cut into small pieces and ground twice using a meat grinder (Waring Consumer Products, East Windsor, NJ) to ensure homogeneity. For the raw samples, about 8 grams of minced meat from each species were weighed into a beaker covered with aluminum foil and 9 fold (w/v, 1 gram raw sample /9 mL extract buffer) saline (0.15M sodium chloride) added to the mashed sample to extract the soluble proteins. For the cooked samples, about 8 grams of minced meat from each species were weighed into a beaker covered with aluminum foil and sealed with autoclave tape, then heated in a boiling water bath for 8 min at 100°C. After cooling to room temperature, each cooked sample was mashed into fine particles. Then 2 fold (w/v, 1 gram cooked sample/ 2 mL extract buffer) of the extraction buffer (0.15 M sodium chloride) was added to the mashed samples to extract the soluble thermostable proteins. All samples were held overnight at 4°C, then centrifuged, dispensed and stored in the same manner as the cooked seafood samples described above.

3.2.3 Preparation of Other Protein Extracts: Nonmeat Protein Extracts

The nonmeat proteins used in this study included BSA, egg albumin, gelatin, NFDM, soy protein concentrate and whey. About 2 grams of each sample were weighed into a beaker and mixed with 2 fold (w/v, 1 gram sample/ 2 mL extract buffer) of 0.15 M sodium chloride. Either after (cooked samples) or without (raw samples) heating the mixture in a boiling water bath for 8 min at 100°C, each sample was mashed with 3 fold (w/v, 1 gram sample/ 2 mL extract buffer) of 0.15 M sodium chloride. The sample extracts were then centrifuged, dispensed and stored in the same manner as the cooked seafood samples described above. The protein concentration of each extract was determined using the Protein Assay Kit II (Bio-Rad). BSA was used as the protein standard.

3.2.4 Preparation of Selected Fish Protein Extracts Stored for Different Lengths of Time

Selected fish species (yellow fin tuna, swordfish and amberjack) were stored at -20°C to study the storage effects. Protein extracts of each sample stored for three different lengths of time

(eleven mouths, eight months, six months, three months, and freshly purchased) were prepared. The samples were extracted, centrifuged, dispensed and stored in the same manner as the cooked seafood samples described above. The protein concentration of each extract was determined using the Protein Assay Kit II (Bio-Rad). BSA was used as the protein standard. Additionally, fish extracts prepared from freshly purchased yellow fin tuna, swordfish and amberjack were also stored for different lengths of time (4 month, 2 month and 1 month) at -20°C and then tested.

3.2.5 Preparation of Protein Extracts from Representative Fish with Different Processing Methods

Selected processed (salted or smoked) fish and their unprocessed counterparts (Table 4) were used to prepare extracts. As the salt in the processed fish may interfere with the accuracy of the assay, it was necessary to first desalt the processed fish. This was done by soaking the processed fish samples in 50mL DDI water for 2 hours at 4°C, changing the water every 10 min in the first hour and then every 20 min in the second hour. The unprocessed samples did not require desalting. All samples were then cut into small cubes, 5 grams each, for raw or cooked sample extraction. The samples were extracted, centrifuged, dispensed and stored in the same manner as the cooked seafood samples described above. The protein concentration of each extract was determined using the Protein Assay Kit II (Bio-Rad). BSA was used as the protein standard.

3.2.6 SDS–Polyacrylamide Gel Electrophoresis and Western Blot

To study the protein profile of the seafood samples and examine the molecule weight of the target antigenic protein of MAbs 2G9, 8F5 and 2F3, selected cooked fish protein extracts were separated by means of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking gel and 14% separating gel) in a Mini-Protean III electrophoresis cell (Bio-Rad) (Laemmli 1970). The running buffer was 25 mM Tris buffer (pH 8.3) containing 192 mM glycine and 0.1% SDS (g/ml). After running at 200 volts for about 45 min at room temperature, the gels were stained with the EZBlue Gel Staining Reagent.

To further examine the antigenic protein in the cooked form of the extracts, the separated protein bands in unstained gels were transferred to supporting nitrocellulose membranes using the Mini Trans-Blot electrophoretic transfer cell according to the method of Towbin and others (1979). The transfer buffer was 25 mM Tris buffer (pH 8.3) containing 192 mM glycine, 0.1% SDS (g/ml) and 20% methanol (ml/ml). The running condition was 100 volts for 1 h with the cooling unit. After transferring, the membranes were incubated in the blocking solution (20 mM TBS containing 1% BSA [g/ml]) for 2 h at room temperature, and then incubated overnight at 4ºC with monoclonal antibodies diluted in the antibody buffer (TBS containing 1% BSA [g/ml] and 0.05% Tween 20 [ml/ml]). The membranes were then incubated for 2 h with anti-IgG-AP conjugate (Bio-Rad) diluted 1:3,000 (ml/ml) in the antibody buffer. Staining was performed using the AP conjugate substrate kit (Bio-Rad) following the manufacturer's instructions. Between each step, the membranes were washed several times in the washing buffer (20 mM TBS containing 0.05% Tween 20 [TBST, ml/ml]). The Precision Plus Protein Kaleidoscope standards (Bio-Rad) were used for estimating molecular weights on the gels and blots.

3.2.7 Indirect Non-Competitive Enzyme-Linked Immunosorbent Assay (iELISA)

Indirect non-competitive ELISA(iELISA) was performed to: 1) test species specificity and cross-reactivity of MAbs 8F5, 2G9, and 2F3 to sample extracts from fish, meats, and common protein additives; 2) develop a new test assay using pooled MAb 8F5, 2G9 and 2F3 (1:1:1); and 3) investigate the effect of storage and processing on fish sample assays. The species specificity and cross-reactivity of MAb 8F5, MAb 2G9 and MAb 2F3 were confirmed by testing against various raw and cooked sample extracts using iELISA. The pooled MAb 8F5, MAb 2G9 and MAb 2F3 (1:1:1) were used against the same set of samples using iELISA. The effect of storage and processing on the testability of selected fish products were also examined by iELISA.

Sample extracts were properly diluted in 50 mM carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6) and coated onto a 96-well flat bottom polystyrene high bind microplate (Costar 9018) (Fisher Scientific, Fair Lawn, NJ) at 2 ug/100ul(well) , then incubated at 37°C for 2 h. After three washing steps with the washing buffer (PBS containing 0.05% Tween 20 [PBST, ml/ml], pH 7.2), the remaining binding sites were blocked with the blocking

buffer (PBS containing 1% BSA [g/ml], pH 7.2) for 2 h at 37°C. After two washes, monoclonal antibodies diluted in antibody buffer (PBST containing 1% BSA [g/ml], pH 7.2) were added to the plate and incubated at 37°C for 2 h. After another three washes, 100 µl/well of anti-IgG-HRP conjugate (Sigma-Aldrich) diluted 1:3,000 in the antibody buffer were added to the plate and incubated at 37°C for 2 h. Following five washing steps, the color was developed by adding 100 µl/well of 0.4 mM ABTS substrate solution (0.22 mg/ml of ABTS in 50 mM phosphate-citrate buffer [50 mM Na_2HPO_4 and 25 mM citric acid, pH 5.0] containing 0.15 µl/ml of H_2O_2) followed by incubation at room temperature for 30 min. The reaction was stopped by adding 100 µl/well of citric acid. The absorbance was read using a PowerWave XS microplate reader (Bio-Tek Instruments, Inc. Winooski, VT) at 410 nm.

3.2.8 Dot Blot

The species specificity and cross-reactivity of MAb 8F5, MAb 2G9 and MAb 2F3 were confirmed by testing against various raw and cooked sample extracts including fish, meats, common protein additives, fish samples storage in different length, and processed fish samples using indirect dot blot. The effectiveness of pooling MAb 8F5, MAb 2G9 and MAb 2F3 (1:1:1) was confirmed by testing against various raw and cooked sample extracts including meats and common protein additives using dot blot.

One microgram of protein in 5 µl of extract was loaded onto a circular area in the nitrocellulose membrane (Bio-Rad) marked by a template. After washing twice with washing buffer (20 mM TBS containing 0.05% Tween 20 [TBST, ml/ml], pH 7.5), the membrane was removed from the apparatus and incubated in 10 mL blocking solution (20 mM TBS containing 3% BSA [g/ml], pH 7.5) for 1 h at room temperature. The membrane was then incubated overnight at 4^{o}C with monoclonal antibodies diluted in the 5 mL blocking buffer. Between each step, the membrane was washed several times in the washing buffer (20 mM TBS containing 0.05% Tween 20 [TBST, ml/ml]). The membrane was then incubated for 2 h with anti-IgG-AP diluted 1:3,000 (ml/ml) in the antibody buffer (TBST containing 1% BSA [g/ml], pH 7.5). Staining was performed using the AP conjugate substrate kit (Bio-Rad) following the manufacturer's instructions.

3.2.9 Assay Validation

The iELISA and dot blot were first validated according to specificity and sensitivity (Dixon 1998): sensitivity= A/B×100%, where A is the number of samples with a true-positive result and B is the number of samples with a positive result, and specificity= C/D×100%, where C is the number of samples with true-negative result and D is the number of samples with a positive result. Secondly, the assay precision was determined according to the FDA guidance for industry bio-analytical method validation (FDA, 2001). The intra-assay and inter-assay reproducibility (%CV) of iELISA results were examined to provide a statistical measure of the variation between replicate determinations in the same assay (intra-assay variability) and in different assays (inter-assay variability), represented by the coefficient of variation (%CV) (Crowther, 2000). It is generally deemed acceptable if the %CV is lower than 15% (FDA, 2001). Intra-assay and inter-assay %CVs of the assay were computed from different samples. The intra-assay variability was calculated by analysis of duplicates of each sample within a plate. The inter-assay variability was determined in order to show the closeness of individual measurements of the same sample conducted on different days to test the reproducibility of the assay, which was calculated by analysis of duplicate replicates of each sample carried out on three consecutive days (FDA, 2001). Overall accuracy consisted of the combined ability to detect positive and negative samples (overall accuracy= specificity+ sensitivity) (Dixon 1998). This was computed as E/F ×100% where F represents the total number of positive and negative samples tested and E the number of positive and negative samples correctly detected by the assay.

3.2.10 Statistical Analysis

Each sample was tested in duplicate, and each experiment repeated two to three times. Results were analyzed using Microsoft Office Excel 2007, SAS 9.2, and SPSS 12.0 software. One-way analysis of variance (ANOVA) was performed; $P < 0.05$ was considered statistically significant.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Selectivity of Three MAbs Using iELISA and Dot Blot

4.1.1 MAb 8F5 Could Recognize 57 of 63 Commercially Important Fish Samples

The species specificity of these three antibodies was tested against extracts of raw and cooked samples of sixty-three commercially important fin fish species. MAb 8F5 reacted with 57 of the 63 species (the exceptions being yellow fin tuna, blue fin tuna, albacore, escolar, wahoo and swordfish) using both dot blot and iELISA when fish were cooked, as shown in Table 5. Although the antibody MAb 8F5 was produced using heated crude red snapper extract as the immunogen, these antibodies recognize relatively small regions of antigens (epitopes) and can thus on occasion find similar epitopes on related molecules, which forms the molecular basis for cross-reaction (Harlow and Lane 1988). This explains why the antibody, although produced using a specific species of fish as the immunogen, can react with other species.

Among those positively-reacted fish species, MAb 8F5 showed strong immunoreactivity to cooked samples but was negative for raw fish samples, as indicated by both the indirect ELISA and dot blot results (Table 6). Since the immunogen of this antibody was a cooked fish crude extract, it is reasonable to expect strong immunoreactivity with cooked fish. The ability of the antibody to react with the cooked sample indicates that the target antigen is thermo-stable. This was likely due to the exposure of the epitope after heat-induced protein unfolding. Furthermore, the heat stable antigen was concentrated after the heat-labile proteins had been aggregated and precipitated. When loading the same amount of protein, the amount of target antigen in cooked samples is higher than that in raw samples, thus making a stronger signal for the cooked fish sample. Although the raw fish could not be directly detected, it can be detected after heating the raw fish for several minutes.

Some slightly differences were observed between the iELISA and dot blot results (Table 5). Weakly positive fish such as sockeye salmon, farm raised salmon and fresh water bream in the iELISA system showed a strong positive signal in the dot blot system, while strong positive fish such as lady fish, sand perch, pink snapper and cubera snapper in iELISA showed only a weak positive in the dot blot system. This might be due to the different binding capacity of the two systems (i.e. the iELISA plate and nitrocellulose membrane) to coated protein. The coated protein can be absorbed to a polystyrene surface (iELISA) via either hydrophobic interactions or direct covalent binding after passive absorption, but the physiochemical basis of binding of proteins to nitrocellulose (dot blot) is believed to be largely due to hydrophobic interactions. The relatively lower binding capacity of nitrocellulose membrane (Stott, 1989) might partially explain why some fish samples showed only weak signals in dot blot but strong iELISA results, but this does not explain those samples weak in iELISA but strong in dot blot. However, it is important to distinguish between the total amount of molecules that can be bound to the surface and the amounts that can be bound and still remain epitope active. It is possible that the amount of active epitope is different in some fish, especially those that have been cooked with some extent of denaturation.

The antibody 8F5 is sufficiently powerful to recognize most of the commercially important fin fish, although it failed to recognize tuna (yellow fin tuna, blue fin tuna, albacore), wahoo, escolar and swordfish, as shown in Table 5. Among those six species of fish with a negative reaction to MAb 8F5, several common characteristics have been observed: 1) these six species have much lower soluble protein concentrations (Table 5) after cooking, which might be due to changes in the protein composition after cooking; and 2) dark muscle fish (all these six) seem to have different allergenicity characteristics to white muscle fish (Kobayashi et al., 2006). It is may therefore be possible to develop MAbs against those dark muscle fish cooked extract to fill this gap.

4.1.2 MAb 2G9 and MAb 2F3 Could Fill the Gap with Minor Cross Reactivity With Other Fish Species

Two MAbs, 2G9 and 2F3, were previously developed by Dr. Hsieh using cooked yellow fin tuna extract and swordfish extract, respectively. The species specificity of MAb 2G9 and MAb 2F3 were tested using raw (Table 6) and cooked samples (Table 5) of sixty-three commercially important fin fish using indirect ELISA and dot blot. These two MAbs (2G9 and 2F3) did indeed react with the fish species that MAb 8F5 failed to pick up. MAb 2G9 reacted strongly with tuna species (yellow fin tuna, blue fin tuna, and albacore), escolar, and wahoo, but not with most of the fish species positive to MAb 8F5 in both iELISA and dot blot. Mahi-mahi, which was positive to MAb 8F5, weakly reacted with MAb 2G9, as shown by iELISA. MAb 2F3 reacted strongly with swordfish and escolar in both iELISA and dot blot.

These two MAbs (2G9 and 2F3) showed weak reactivity with raw fish samples compared to cooked samples using both indirect ELISA and dot blot, the same trend as that of MAb 8F5. All three MAbs used cooked fish extract as the imunogen for antibody production, thus it is reasonable to expect strong immunoreactivity with cooked fish but only a relatively weak reaction with raw fish. That these antibodies react with cooked samples indicates that their target antigens are thermo-stable. There was also some variation between the iELISA and dot blot assays. All three tuna species and wahoo produced weak positive reactions in iELISA but only yellow fin tuna weakly reacted in dot blot using MAb 2F3. This is understandable, as the variation in the surface characteristics of the two systems for absorbing and immobilize protein make the epitope exposure different for each.

All of the fish positively reacted with MAb 2F3 and MAb 2G9 in both iELISA and dot blot, have similar soluble protein concentration (<0.45 mg/ml, a 96% decrease from the uncooked samples) after cooking; this might indicate their similar precipitation characteristics for most proteins and the remaining higher concentration of antigenic protein, or it might suggest that certain protein structures in these fish are unique due to the exposure of the epitope of the antigenic proteins after cooking. The fish (>1.8 mg/ml protein concentration after cooking, a 80% decrease from the uncooked samples) that negatively reacted with MAbs 2F3 and 2G9 may contain proteins

similar to the antigenic proteins; however, either these have been precipitated or cooking failed to expose the epitope.

4.1.3 All Three MAbs Had No Cross Reactivity with Non Fish Samples

Commercial food products may contain non fish meat and food additives (Figure 3). This experiment thus sought to determine if any of these antibodies have cross reactivity with any of the non-fish proteins likely to be present in food products. The non-fish proteins were categorized into two groups, namely protein food additives (BSA, non-fat dry milk, gelatin, soy protein and egg albumin) and non-fish meat proteins (raw and cooked meat from non-fin fish, shell fish, poultry, and mammalian animals). BSA was used as the blocking buffer and the antibody buffer due to its clean background. This part of the study also sought to confirm if any or all three of the antibodies cross-reacted with BSA, gelatin or nonfat dry milk, which are all possible choices as a blocking buffer, and hence whether it was acceptable to continue using BSA as the blocking buffer. None of the three antibodies (MAbs 8F5, 2G9 and 2F3) cross-reacted with any of the food additive proteins tested nor with any of the heat-treated meat samples tested. Thus, BSA was maintained as the blocking buffer and antibody buffer for all the subsequent indirect ELISA and dot blot assays.

In summary, all three MAbs reacted strongly with cooked fish and were not cross reactive with the non-fin fish proteins. MAb 8F5 reacted strongly with 57 of the 63 commercially important fin fish, with the exceptions being yellow fin tuna, blue fin tuna, albacore, escolar, wahoo and swordfish. MAb 2F3 reacted strongly with swordfish and escolar, and weakly with yellow fin tuna, blue fin tuna, albacore, and wahoo. MAb 2G9 reacted strongly with tuna (yellow fin, blue fin and albacore), escolar, and wahoo. Pooling these three together makes the detection of all 63 commercially important fin fish possible. To better understand and apply these MAbs, further characterization could provide further information that would explain their binding characteristics.

4.2 Characterization of the Antigenic Proteins of MAbs 8F5, 2G9 and 2F3

4.2.1 SDS-PAGE Showed Different Patterns Among Selected Fish Samples

Cooked protein extracts of swordfish, yellow fin tuna, blue fin tuna, albacore, escolar, wahoo, mahi-mahi and amberjack were resolved by 12% SDS-PAGE and visualized by coomassie staining (Figure 4). These selected fish samples include all MAb 2G9 and MAb 2F3 positive fish species and two representatives of MAb 8F5 fish (mahi-mahi and amberjack). Since MAb 8F5 is cross reactive with a wide range (57) of fish species, the protein profile of its positive fish species can be used as a reference to compare with the MAb 8F5 negative fish that react with either MAb 2G9 or MAb 2F3. The protein extracts of these species established different protein patterns (Fig. 4), with substantial amounts of thermal-stable proteins present. Patterns of muscle proteins are affected by physiological factors, environment, seasons, stress, starvation, breeding season and migration (Ando et al., 1985; Gomez et al., 2000; Ladrat et al., 2000; Delbarrel-Ladrat et al., 2006) among different species of fish.

Several similarities can be observed, however. One major group of proteins (appearing as a 36 kD band on the gel) with two bands appears only in mahi-mahi and amberjack, which positively reacted with MAb 8F5 from both indirect ELISA and dot blot. These bands may be the tropomysin isoforms, which have been reported to be thermally stable (Naqpal et al., 1989). Similar protein bands from 30-150 kD are present in fish species that were positive to MAb 2F3, which might be due to degraded actin, myosin, and troponin filaments (Xiong, 1997) because myofibrillary proteins (myosin, actin, tropomyosin and actomyosin) have been reported to constitute 70%-80% of fish muscle proteins (Huss, 1995). Interestingly, a protein band around 25 kD appeared in all species except amberjack. Based on its molecular weight, this could be either the myosin light chain (Levitsky et al., 1991) or troponin-I, which has been reported to be a thermal stable protein. Judging from the thermal stable patterns, species differences could be deduced. However, there is lack of evidence that clearly indicates how protein patterns related to these species reacte with different antibodies.

4.2.2 Western Blot Demonstrated Different Antigenic Proteins Identified by Three MAbs

Following SDS-PAGE, protein bands were transferred to nitrocellulose membranes to immune-blot with MAbs 2G9, 2F3 and 8F5. As previously mentioned, MAb 8F5 was developed against crude cooked red snapper soluble protein extracts. The western blot results (Figure 5) show that MAb 8F5 recognized 36-kDa antigenic proteins specific to mahi-mahi and amberjack, which is in accord with the iELISA and dot blot results.

MAb 2G9 recognized a major protein band around 25kD (Figure 6) and several minor bands among yellow fin tuna, blue fin tuna, escolar, wahoo, and mahi-mahi. All species tested in western blot except mahi-mahi showed consistently positive results from iELISA and dot blot. Interestingly, mahi-mahi not only positively reacted with MAb 8F5, but was also cross reactive with MAb 2G9, which was shown in both iELISA and dot blot. This is possibly due to the SDS buffer's effects on the unfolding of proteins, which extends the peptide to a linear form, and thus exposes hidden epitopes to react with the paratopes of MAb 2G9. This also demonstrates that the antigenic protein of MAb 2G9 could be a protein universally present in fish muscle, although its expression of folding will differ slightly among species. Troponin I might be a possible candidate for antigenic protein due to several studies finding anti-troponin antibodies being produced by injection of crude protein extracts and troponin I is reported to be a thermal-stable species marker (Chen and Hsieh 2001). Research needs to be done to further characterize the antigenic protein of MAb 2G9.

MAb 2F3 binds to multiple bands in the range 30-150 kD (Figure 7) in swordfish, escolar, wahoo, yellow fin tuna, blue fin tuna and albacore. This finding implies the antigenic proteins might be degraded muscle proteins available in fish species but encoded slightly differently as a result of the evolution process. The myosin light chain, a subunit of actin, or the myosin or troponin isoforms were possible antigens due to the closeness of the molecular weight range (Xiong, 1997), however, solid proof needs to be established to confirm this assumption. This blot also further confirmed MAb 2F3's cross reactivity with some fish species that also positively reacted to MAb 2G9 in iELISA and dot blot. The escolar showed a minor band around 30 kD when blotted with MAb 2G9, which might also indicate a slight overlapping of epitopes between MAb 2G9 (30 kD, minor band) and MAb 2F3 antigens (30kD).

The western blot results demonstrated that all three MAbs, 2G9, 8F5 and 2F3, have their own antigenic proteins with different target fish species and few overlapping epitopes, giving coverage for the complete range tested and showing great promise for the detection of fish species as a whole.

4.3 Detection of All 29 Selected Common Food Fish Species Using Pooled MAbs

Twenty nine representative fish, selected from MAb 8F5 negative samples, weak positive samples, strong positive samples, were tested using pooled MAbs (2G9, 2F3, 8F5) in iELISA and dot blot. In order to contrast the effect on immunoreactivity of using individual single MAb and pooled MAbs, all selected samples were also tested for immunoreactivity with the same amount of each individual MAb. The results (Table 7) show that the pooled MAbs retained the same or higher immunoreactivity compared to their individual use in both iELISA and dot blot. This indicates little interference or inhibition among the MAbs when added together. The immunoreactivity of the fish samples showed a very strong positive reaction with one MAb and a weak positive in another. For example, mahi-mahi reacted strongly positively to MAb 8F5 but weakly positively to MAb 2G9, with a dramatical increase in immunoreactivity in iELISA using the pooled MAbs.

Escolar showed a very strong positive to MAb 2G9 and a strong positive to MAb 2F3, retaining a similar strength immunoreactivity to MAb 2G9 when using pooled MAbs. This may be partially due to the epitope similarity of their antigens. These results suggest that the epitopes may be shared between MAb 2G9 and MAb 2F3, so MAb 2G9 competed with the MAb 2F3 binding site with the saturated amount of antigen. In contrast, MAb 2G9 and MAb 8F5 may not share any regions on their epitopes, thus making the immunoreactivity of mahi-mahi stronger than the single use of each MAb. All these reacted accorded to the western blot patterns of the three MAbs. No species has been found to react with both MAb 8F5 and MAb 2F3, thus the characteristics of their epitope similarity could not be observed. Furthermore, the results of using pooled MAbs in dot blot and iELISA exhibited some of the same differences as in the individual

MAb testing: sockeye salmon and farm raised salmon both continued to show a strong positive in dot blot and a weak positive in iELISA.

The uncooked sample extracts of selected fish were also examined and showed no signal stronger than the negative control (Table 8). The non-fish samples, including land animal, shell fish and common food additive proteins mentioned before, were used to test the effectiveness of the pooled MAbs (Figure 8). No cross reactivity with the non-fish samples was found in either the iELISA or the dot blot. These results were again in agreement with those for the individual MAbs, which was as expected and further demonstrated the specificity and effectiveness of the pooled MAbs for use as a standard reagent.

4.4 Effects of Storage Length, Cooking Time and Processing on the Immunoreactivity of Selected Fish Using iELISA and Dot Blot

4.4.1 Effect of Fish Sample Storage on Immmunoreactivity

Cooked yellow fin tuna, amberjack and swordfish were selected to examine whether storage of the fish muscle samples would affect their reaction to the individual MAbs 2G9, 8F5, and 2F3. All these species were selected in this experiment due to their positive reaction to their representative specific MAb. Freshly purchased yellow fin tuna fillet was cut into 5 inch cubes and stored in -20ºC freezers for 0 days, 8 months, and 11 months and then water soluble proteins extracted using 0.15 M saline. Saline extracts of amberjack stored for 0 days, 3 months, 6 months and swordfish stored for 0 days, 6 months, 11 months were prepared in the same manner. The results showed that although these three fish samples showed a slight decrease in immunoactivity in both iELISA and dot blot, the yellow fin tuna and swordfish could be detected even after 11 months of storage using MAb 2G9 and 2F3, respectively. A sharp decline in the immunoreactivity of amberjack with MAb 8F5 was observed; although a strong positive signal after three months of storage was observed, the immunoreactivity after six months of storage was undetectable by both iELISA and dot blot. This general trend of decreasing immunoactivity over storage was expected because soluble protein is known to slowly degrade under frozen storage, either reversibly or irreversibly (Shaviklo et al, 2012), which alters the protein structure and thus decreases the chance of the antigen being detected by the antibody. MAb 8F5 may not have been

able to detect amberjack after 6 months of storage due to the following reasons: 1) the conformation of MAb 8F5 antigen changed, resulting in an epitope that was undetectable after proteolysis and oxidation; 2) the antigen was more sensitive and susceptible to degradation than other fish species after long-term storage, possibly due to factors such as mechanical damage due to ice formation and ice crystal damage proteins during freeze cycle-recrystalization; 3) dehydration (water-ice) led to increases in the hydrophobic side chains, thus altering the epitope conformation; and/or 4) the antigen distribution in the amberjack harvested in the spring and fall were different due to the diet and environment, and other MAb 8F5 positive fish may not follow a similar trend. These overall results indicate that the immunoreactivity of these three antibodies (MAb 2G9, 2F3, 8F5) remain capable of recognizing their target fish species that have been stored in a freezer for up to 3 months or longer in some cases.

4.4.2 Effect of Fish Extract Storage on Immmunoreactivity

It is common in research and inspection laboratories that extracted samples may be stored in freezers for months before testing or for retesting. There is little information available in the literature regarding the frozen storage of sample extracts on their solubility and antigenicity. Cooked yellow fin tuna, amberjack and swordfish extracts stored in a -20ºC freezer for different periods of times (the same as the fish samples described in the section 4.4.1) were examined using MAb 2G9, MAb 8F5 and MAb 2F3, individually. All except the 6-month stored amberjack could be detected after months of storage using both iELISA and dot blot. A decrease in immunoreactivity was observed in all three for their representative fish and this was also expected due to the freezing conditions, which altered the protein structure as described above. Although these fish extracts showed similar trends to the extracts of stored fish muscles, significantly ($p<0.05$) lower immunoreactivity was observed. The difference between stored fish meat and fish extract can be explained by the variation in the soluble protein characteristics between muscle tissue (fish meat) and saline buffer (fish extract) under frozen storage. The simple saline buffer system seemingly suffers more conformation structure loss of antigenic protein, possibly due to its interaction with soluble protein which tends to degrade much earlier than in fish muscle tissue. Under the same period of frozen storage, fish meat could achieve slightly better outcomes than extracts using MAbs 8F5, 2G9 or 2F3. However, most sample

extracts remain detectable; indicating that these three antibodies (MAb 2G9, 2F3, 8F5) are all valid for use with samples that have undergone frozen storage for months.

4.5 Effects of Processing on the Immunoreactivity of Selected Fish Using the Developed Assay

4.5.1 Heating

Heating is a common way to cook fish, and the cooking time varies for different dishes and processing methods. Heat-processing treatments often cause protein denaturation that can lead to a loss of protein solubility. As expected, the reduced solubility after heating depends on the severity and denaturation of processing (Sathe and Roux 2006). Little research has been reported in terms of detectability after different lengths of cooking. Three representative fish, yellow fin tuna, amberjack, and swordfish, were cooked in 100^{o}C boiling water for 0 min, 1 min, 2 min, 4 min, 6 min, 8 min and then examined to verify the affinity of MAb 2G9, 8F5 and 2F3, respectively. All fish tested showed an increase of immunoreactivity though 0 min to 6 min, and then remained stable from 6 min to 8 min in both the iELISA and dot blot assays. These results not only indicate the increase in the amount of antigen after cooking, but also showed that 6 min is the time needed for a thorough antigen exposure in a stovetop cooking system. The immunoreactivity in the boiling water was relatively low from 0 min -2 min and remained stable from 6-8 min, which might suggested the first 6 minutes of heat helps to expose the epitopes, and the durable architecture of the epitopes, in terms of their resistance to denaturation.

However, there were some variations among the three fish. Amberjack showed no signal until four minutes of cooking in both iELISA and dot blot; swordfish showed a positive signal after two minutes of cooking in both iELISA and dot blot; yellow fin tuna showed no signal until four minutes of cooking in iELISA but was positive after two minutes of cooking in dot blot. These differences could be explained by the characteristic of the antigens in the different fish against the three antibodies. The swordfish antigen has a wide range of multiple bands (shown in western blot results) that recognize MAb 2F3 (30kD-150kD), and thus degraded proteins with epitopes were formed throughout the entire heating process.

Yellow fin tuna could be recognized by MAb 2G9 in the dot blot system after only one minute cooking (Figure 12); however, three more minutes heating was needed for the first identification in the iELISA system (Figure 13-A). The antigenic proteins of MAb 2G9 belong to a lower molecular catalog (<37 kDa), and therefore may be exposed more slowly after a period of cooking. The nitrocellulose membrane (dot blot) system seems to deal more subtly with the structure changes in the antigenic protein, thus resulting in a more sensitive recognition of MAb 2G9 than the polystyrene in the ELISA plates during heating. However, amberjack could only been detected after four minutes cooking in both iELISA (Figure 13-B) and dot blot (Figure 12) using MAb 8F5.

The immunoreactivity patterns of the three individual MAbs to their representative fish using dot blot and iELISA further indicates their different characteristic. However, all three needed a certain heating time to successfully detect the fish samples. These further demonstrate the effectiveness of these MAbs for fish detection under heat treatment, which makes it possible for them to be pooled together to explore the complicated processed products.

4.5.2 Salting and Smoking

Smoking fish involves burning wood (pyrolysis) to form smoke, in which the three major components of wood, cellulose, hemicellulose and lignin, are broken down by heat (Rozum 2008). Smoking, one of the oldest preservation methods, combines the effects of salting, drying, and heating. The major steps in the preparation of smoked fish are salting (bath or injection of liquid brine or dry salt mixture), cold smoking, cooling, packaging (air/vacuum or modified), and storage. Typically, fish can be either cold smoked (28–32°C) or hot smoked (70–80°C). Cold smoking does not cook the flesh, coagulate the proteins, inactivate food spoilage enzymes, or eliminate the food pathogens, and hence refrigerated storage is necessary until consumption.

The results (Figure 14) revealed that salt processing, with a salt content of anywhere from 20% to 50%, could not affect the stability of the antigenic protein. This indicates that in the salting/drying process, the presence of NaCl may lead to a significant increase in protein aggregation and result in irreversible degradation and thus provides a way to expose the

antigenic protein epitope without heating. Salting/drying processing may also involve some degree of heating, for example sun exposure. The salted mackerel showed interesting results that turns to react to all MAbs weakly which heated mackerel only reacted to MAb 8F5. This might be an indication that the salt process can alter certain protein structures unpredictably. Of all the dot blot results for salted fish, the 8 min cooked sample showed relatively less immunoreactivity compared to the non-cooked samples, while the iELISA showed the opposite.

For smoked carp, salmon and Atlantic herring, the signal was detectable with or without heating for 8 minutes in boiling water. This indicates the stability of antigenic protein retained in serious conditions with salt, cold/hot smoke and boiling water for long periods of time. A slight decrease in the immunoreactivity has been observed when smoked fish was boiled for 8 minutes, suggesting repeated heating may degrade or aggregate antigenic proteins. Interestingly, the smoked salmon (Figure 14), which showed negative reactivity in iELISA, positively reacted with pooled MAbs. This may further indicate that the nitrocellulose membrane (dot blot) system is more sensitive than iELISA when detecting fish products under processing. However, smoked mackerel showed no signal in either iELISA or dot blot, whether heated or non-heated. The reason for this is unknown, although it is possible that after long-term (unknown) smoking, high concentrations of salt or aroma compounds have altered its epitope structure.

Overall, all three MAbs, either individually or pooled, effectively detected fish ingredients under heat, salt and smoke processing in both iELISA and dot blot assays. The dot blot system, which is both simple and user-friendly, showed no weaker sensitivity compared to iELISA in commercial processed fish detection

4.6 Validation of the iELISA and Dot Blot Assays

Reproducibility is used to define and asess the variability between replicate determinations in the same assay (intra-assay variability) and in different assays (inter-assay variability). It is represented as the coefficient of variation (CV) computed as described earlier (Dixon 1998). This was done by using data from the experiments with both individual MAbs and the pooled MAbs

for the fish samples in iELISA. The assay had an intra assay variability of 0.15%-0.80% (Table 9), and an inter assay variability of 0.44 %-0.50% (Table 10).

Sensitivity is the ability of a test to detect positive samples as positive and is computed as A/B ×100%, where B (586) was the number of positive samples tested and A (582) the number of positive samples that the test was able to correctly detect as positive (Dixon 1998). All commercially important fish samples (raw, cooked, smoked, frozen storage, heat, salted, salted and heated, smoked and heated) using individual MAbs (2G9,2F3 and 8F5) and pooled MAbs in iELISA and dot blot has been include. The sensitivity of the dot blot and iELISA assay using individual MAbs thus had sensitivities of 99.2% and 99.3% (Table 11), respectively. The pooled MAbs achieved 100% in both the dot blot and iELISA assays.

Specificity defined for this iELISA here is the ability of a qualitative test to detect negative samples as negative and was computed as C/D × 100% where D (732) was the number of negative samples tested and C (732) was the number of negative samples that test was able to correctly detect as negative. This was done using samples as the sensitivity test. The assay achieved a 100% (Table 12) specificity in tested samples using individual MAbs and pooled MAbs in both iELISA and dot blot.

The overall accuracy is the combined ability of the test to correctly detect positive and negative samples (overall accuracy=specificity + sensitivity) (Dixon 1998). This was computed as E/F ×100% where F (1318) was the total number of positive and negative sample tested and E (1314) the number of positive and negative samples correctly detected by the assay. The assay had an overall accuracy of 99.7% (Table 13).

CONCLUSIONS

Two new sensitive and specific immunoassays, iELISA and consumer friendly dot blot, utilizing pooled MAbs for fish protein detection were established. Three MAbs (8F5, 2G9 and 2F3) were used to study their immunoreactivity individually against sixty three commercially important fin fish and then combined to form a pooled MAb set against twenty-nine selected fish. MAb 8F5 could react with 57 of the 63 species tested, while between them 2G9 and 2F3 could react with all six of the remaining species. Pooling the three MAbs made it possible to detect all twenty nine of the selected fish samples with no cross reactivity with non-fish samples. These three MAbs bonded with 36kD, 25kD, 30-150kD thermal stable antigenic proteins in red snapper, yellow fin tuna, and swordfish, respectively. All three MAbs, individually or pooled, were able to detect fish products under cooking, storage, salted and smoked conditions.

Both assays exhibited excellent reproductively, sensitivity, specificity and appear to be very suitable for the qualitative detection of fish proteins, whether raw, cooked, and processed. The dot blot, as a consumer friendly assay, has the potential for rapid screening of large numbers of samples in the field.

APPENDIX A

TABLES

Table 4 Processed fish sample information

Products	Company	Ingredients
Wild caught Smoked Mackerel	Fish and seafood dept 6035 Peachtree Rd bldg B Doraville Ga 30360	Mackerel, salt, natural smoke
Atlantic smoked mackerel	Mr. Fish Brand Imported by: Euro gourmet foods 2406 Tech center pkwy suite 400 Lawrenceville Ga 30043	Mackerel, salt, natural smoke
Salted butterfish, farm raised	Grand Bk Corp 4708 Grand Ave Maspeth NY 11378	Butterfish, Salt
Smoked carp	B and K grocery store 1265 Lee St SW # C, Atlanta, GA30310	Carp, salt, natural smoke
Salted Codfish	Cristobal Brand Distributed by: Canadian Fish exporters, inc Watertown, MA 02471-0411	Codfish, salt Packaged: 55% salt DV Prepared: 26% salt DV
Wild Alaska sockeye smoked salmon, traditional flavor	Echo Falls Distributed by: Ocean Beauty Seafood's LLC Seattle WA 98119	Sockeye salmon, salt, brown sugar, natural hardwood 27% sodium DV

Table 5 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 against fish species (cooked) using iELISA and dot blot

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Amberjack	*Seriola dumerili*	+++	++	+/-	+	+/-	-
Atlantic croaker	*Micropogonias undulates*	++	+++	-	-	-	-
Basa	*Pangasitus bocourti*	+++	+++	-	-	-	-
Black grouper	*Mycteroperca bonaci*	+++	+++	-	-	-	-
Black rock bass	*Mycteroperca phenax*	+++	+++	-	-	-	-
Black sea bass	*Centropristis striata*	+++	+++	-	-	-	-
Blue cat fish	*Ictalurus furcatus*	+++	+++	-	-	-	-
California sea bass	*Stereolepis gigas*	+++	+++	-	-	-	-
Caribbean red snapper	*Lutjanus purpureus*	+++	+++	-	-	-	-
Cat fish	*Ameiurus catus*	+++	+++	-	-	-	-
Chilean sea bass	*Dissostichus eleginoides*	+++	+++	-	-	-	-
Cod	*Gadus morhua*	+++	++	-	-	-	-
Cubera snapper	*Lutjanus cyanopterus*	++	+	-	-	-	-
Farm raised salmon	*Oncorhynchus gorbuscha*	+	+++	-	-	-	-

Table 5 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Fresh water breams	*Abramis brama*	+++	+++	-	-	-	-
Gag grouper	*Mycteroperca microlepis*	++	+++	-	-	-	-
Grass porgy	*Calamus arctifrons*	+++	+++	-	-	-	-
Gulf Flounder	*Paralichthys albigutta*	+++	+++	-	-	-	-
Haddock	*Melanogrammus aeglefinus*	+++	+++	-	-	-	-
Halibut	*Hippoglossus Stenolepsis*	+++	+++	-	-	-	-
Hog snapper	*Lachnolaimus maximus*	+++	+++	-	-	-	-
Idaho Rain bow trout (farm raised)	*Oncorhynchus mykiss*	+	+++	-	-	-	-
Key west grunt	*Labrus plumierii*	+++	+++	-	-	-	-
King mackerel	*Scomberomorus cavalla*	+++	+++	-	-	-	-
Lady fish	*Elops saurus*	++	+	-	-	-	-
Lane snapper	*Lutjanus synagris*	+++	+++	-	-	-	-
Mahi mahi	*Coryphaena hippurus*	+++	+++	+	+/-	-	-
Mangrove snapper	*Lutjanus griseus*	+++	+++	-	-	-	-

Table 5 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Mississippi redfish	*Sciaenops ocellatus*	++	+++	-	-	-	-
Mullet	*Agonostomus telfairii*	++	+	-	-	-	-
Nile perch	*Lates niloticus*			-	-	-	-
Ocean perch	*Sebastes alutus*	+++	+++	-	-	-	-
Orange roughy	*Hoplostethus atlanticus*	+++	+++	-	-	-	-
Pig fish	*Orthopristis chrysoptera*	+++	+++	-	-	-	-
Pink snapper	*Pagrus auratus*	++	+	-	-	-	-
Pollock	*Pollachius pollachius*	+++	+++	-	-	-	-
Pompano	*Trachinotus carolinus*	++	+++	-	-	-	-
Red snapper	*Lutjanus campechanus*	+++	+++	-	-	-	-
Red grouper	*Epinephelus morio*	+++	+++	-	-	-	-
Rock bass	*Ambloplites rupestris*	+++	+++	-	-	-	-
Sand perch	*Parapercis millepunctata*	++	+	-	-	-	-
Scamp grouper	*Mycteroperca phenax*	++	+++	-	-	-	-
Sheep head	*Archosargus probatocephalus*	+++	+++	-	-	-	-

Table 5 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Sockeye salmon	*Oncorhynchus nerka*	+	++	-	-	-	-
Southern flounder	*Paralichthys lethostigma*	+++	+++	-	-	-	-
Spanish mackerel	*Scomberomorus maculatus*	+++	++	-	-	-	-
Speckled trout	*Cynoscion nebulosus*	++	++	-	-	-	-
Striped bass	*Morone saxatilis*	++	+++	-	-	-	-
Tilapia	*Oreochromis niloticus*	+++	+++	-	-	-	-
Tra	*Pangasius hypothalamus*	+	+	-	-	-	-
Vermillion snapper	*Rhomboplites aurorubens*	+++	+++	-	-	-	-
Whiting	*Merlangius merlangus*	+++	+++	-	-	-	-
White grunt	*Haemulon plumieri*	++	++	-	-	-	-
Wild cobia	*Rachycentron canadum*	++	+++	-	-	-	-
Wild salmon	*Oncorhynchus gorbuscha*	+++	+++	-	-	-	-
Wolf fish	*Anarhichas lupus*	+++	+++	-	-	-	-
Yellow edge grouper	*Hyporthodus flavolimbatus*	+++	+++	-	-	-	-

Table 5 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Yellow tail snapper	*Ocyurus chrysurus*	+++	+++	-	-	-	-
Yellow fin tuna	*Thunnus albacares*	-	-	+++	+++	++	+
Blue fin tuna	*Thunnus orientalis*	-	-	+++	+++	+	-
Albacore	*Thunnus alalunga*	-	-	+++	+++	+	-
Escolar	*Lepidocybium flavobrunneum*	-	-	+++	+++	++	++
Wahoo	*Acanthocybium solandri*	-	-	+++	+++	+/-	-
Swordfish	*Xiphias gladius*	-	-	-	-	+++	+++

+++: Very strong positive (O.D 415nm higher than 1 or dark purple)
++: Strong positive (O.D 415nm 0.5-0.999 or purple)
+: Weak positive (O.D 415nm 0.2-0.499 or week purple)
+/-: O.D 415nm 0.15 to 0.199 or very week purple stronger color than negative control
-: negative O.D 415 nm lower than 0.15 or no color as negative control
iELISA was loaded 2 ug/100 uL; Dot blog was loaded 1ug/5ul

Table 6 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 against fish species (raw) using iELISA and dot blot

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Amberjack	*Seriola dumerili*	-	-	-	-	-	-
Atlantic croaker	*Micropogonias undulates*	-	-	-	-	-	-
Basa	*Pangasitus bocourti*	-	-	-	-	-	-
Black grouper	*Mycteroperca bonaci*	-	-	-	-	-	-
Black rock bass	*Mycteroperca phenax*	-	-	-	-	-	-
Black sea bass	*Centropristis striata*	-	-	-	-	-	-
Blue cat fish	*Ictalurus furcatus*	-	-	-	-	-	-
California sea bass	*Stereolepis gigas*	-	-	-	-	-	-
Caribbean red snapper	*Lutjanus purpureus*	-	-	-	-	-	-
Cat fish	*Ameiurus catus*	-	-	-	-	-	-
Chilean sea bass	*Dissostichus eleginoides*	-	-	-	-	-	-
Cod	*Gadus morhua*	-	-	-	-	-	-
Cubera snapper	*Lutjanus cyanopterus*	-	-	-	-	-	-
Farm raised salmon	*Oncorhynchus gorbuscha*	-	-	-	-	-	-

Table 6 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Fresh water breams	*Abramis brama*	-	-	-	-	-	-
Gag grouper	*Mycteroperca microlepis*	-	-	-	-	-	-
Grass porgy	*Calamus arctifrons*	-	-	-	-	-	-
Gulf Flounder	*Paralichthys albigutta*	-	-	-	-	-	-
Haddock	*Melanogrammus aeglefinus*	-	-	-	-	-	-
Halibut	*Hippoglossus Stenolepsis*	-	-	-	-	-	-
Hog snapper	*Lachnolaimus maximus*	-	-	-	-	-	-
Idaho Rain bow trout (farm raised)	*Oncorhynchus mykiss*	-	-	-	-	-	-
Key west grunt	*Labrus plumierii*	-	-	-	-	-	-
King mackerel	*Scomberomorus cavalla*	-	-	-	-	-	-
Lady fish	*Elops saurus*	-	-	-	-	-	-
Lane snapper	*Lutjanus synagris*	-	-	-	-	-	-
Mahi mahi	*Coryphaena hippurus*	-	-	-	-	-	-
Mangrove snapper	*Lutjanus griseus*	-	-	-	-	-	-

Table 6 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Mississippi redfish	*Sciaenops ocellatus*	-	-	-	-	-	-
Mullet	*Agonostomus telfairii*	-	-	-	-	-	-
Nile perch	*Lates niloticus*	-	-	-	-	-	-
Ocean perch	*Sebastes alutus*	-	-	-	-	-	-
Orange roughy	*Hoplostethus atlanticus*	-	-	-	-	-	-
Pig fish	*Orthopristis chrysoptera*	-	-	-	-	-	-
Pink snapper	*Pagrus auratus*	-	-	-	-	-	-
Pollock	*Pollachius pollachius*	-	-	-	-	-	-
Pompano	*Trachinotus carolinus*	-	-	-	-	-	-
Red snapper	*Lutjanus campechanus*	-	-	-	-	-	-
Red grouper	*Epinephelus morio*	-	-	-	-	-	-
Rock bass	*Ambloplites rupestris*	-	-	-	-	-	-
Sand perch	*Parapercis millepunctata*	-	-	-	-	-	-
Scamp grouper	*Mycteroperca phenax*	-	-	-	-	-	-
Sheep head	*Archosargus probatocephalus*	-	-	-	-	-	-

Table 6 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Sockeye salmon	*Oncorhynchus nerka*	-	-	-	-	-	-
Southern flounder	*Paralichthys lethostigma*	-	-	-	-	-	-
Spanish mackerel	*Scomberomorus maculatus*	-	-	-	-	-	-
Speckled trout	*Cynoscion nebulosus*	-	-	-	-	-	-
Striped bass	*Morone saxatilis*	-	-	-	-	-	-
Tilapia	*Oreochromis niloticus*	-	-	-	-	-	-
Tra	*Pangasius hypothalamus*	-	-	-	-	-	-
Vermillion snapper	*Rhomboplites aurorubens*	-	-	-	-	-	-
Whiting	*Merlangius merlangus*	-	-	-	-	-	-
White grunt	*Haemulon plumieri*	-	-	-	-	-	-
Wild cobia	*Rachycentron canadum*	-	-	-	-	-	-
Wild salmon	*Oncorhynchus gorbuscha*	-	-	-	-	-	-
Wolf fish	*Anarhichas lupus*	-	-	-	-	-	-
Yellow edge grouper	*Hyporthodus flavolimbatus*	-	-	-	-	-	-

Table 6 – continued

Fish species	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3	
		iELISA 1 to 20	Dot blot 1 to 10	iELISA 1 to 3	Dot blot 1 to 5	iELISA 1 to 3	Dot blot 1 to 5
Yellow tail snapper	*Ocyurus chrysurus*	-	-	-	-	-	-
Yellow fin tuna	*Thunnus albacares*	-	-	-	-	-	-
Blue fin tuna	*Thunnus orientalis*	-	-	-	-	-	-
Albacore	*Thunnus alalunga*	-	-	-	-	-	-
Escolar	*Lepidocybium flavobrunneum*	-	-	-	-	-	-
Wahoo	*Acanthocybium solandri*	-	-	-	-	-	-
Swordfish	*Xiphias gladius*	-	-	-	-	-	-

+++: Very strong positive (O.D 415nm higher than 1 or dark purple)
++: Strong positive (O.D 415nm 0.5-0.999 or purple)
+: Weak positive (O.D 415nm 0.2-0.499 or week purple)
+/-: O.D 415nm 0.15 to 0.199 or very week purple stronger color than negative control
-: negative O.D 415 nm lower than 0.15 or no color as negative control
iELISA was loaded 2 ug/100 uL; Dot blog was loaded 1ug/5ul

Table 7 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 and three pooled MAbs against selected fish (cooked) using indirect ELISA and dot blot

Market name	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3		MAbs (8F5 +2F3 +2G9) 1:1:1	
		iELISA 1:20	Dot blot 1:10	iELISA 1:3	Dot blot 1:5	iELISA 1:3	Dot blot 1:5	iELISA	Dot blot
Yellow fin tuna	*Thunnus albacares*	-	-	+++	++	++	+/-	+++	+++
Blue fin tuna	*Thunnus orientalis*	-	-	+++	+++	+	-	+++	+++
Albacore	*Thunnus alalunga*	-	-	+++	+++	+	-	+++	+++
Escolar	*Lepidocybium flavobrunneum*	-	-	+++	+++	++	++	+++	+++
Wahoo	*Acanthocybium solandri*	-	-	+++	+++	+	-	+++	+++
Swordfish	*Xiphias gladius*	-	-	-	-	+++	+++	+++	+++
Amberjack	*Seriola dumerili*	+++	++	-	-	+/-	-	++	++
Mahi-mahi	*Coryphaena hippurus*	+++	+++	+	-	-	-	+++	+++
Spanish markerel	*Scomberomorus maculatus*	+++	++	-	-	-	-	+++	++
California basa	*Stereolepis gigas*	+++	+++	-	-	-	-	+++	+++

Table 7 – continued

Market name	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3		MAbs (8F5 +2F3 +2G9) 1:1:1	
		iELISA 1:20	Dot blot 1:10	iELISA 1:3	Dot blot 1:5	iELISA 1:3	Dot blot 1:5	iELISA	Dot blot
Red snapper	*Lutjanus campechanus*	+++	+++	+/-	-	-	-	+++	+++
Wild salmon	*Oncorhynchus tshawytscha*	+++	+++	-	-	-	-	+++	+++
Tilapia	*Oreochromis mossambicus*	+++	+++	-	-	-	-	+++	+++
Cod	*Gadus macrocephalus*	+++	++	-	-	-	-	+++	++
Seawolf	*Anarhichas lupus*	+++	+++	-	-	-	-	+++	+++
Pollock	*Pollachius virens*	+++	+++	-	-	-	-	+++	+++
Catfish	*Ictalurus punctatus*	+++	+++	-	-	-	-	+++	+++
Whiting	*Merlangius merlangus*	+++	+++	-	-	-	-	+++	+++
Basa	*Pangasius bocourti*	+++	+++	-	-	-	-	+++	+++

Table 7– continued

Market name	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3		MAbs (8F5 +2F3 +2G9) 1:1:1	
		iELISA 1:20	Dot blot 1:10	iELISA 1:3	Dot blot 1:5	iELISA 1:3	Dot blot 1:5	iELISA	Dot blot
Cubera snapper	*Lutjanus cyanopterus*	++	+	-	-	-	-	++	+
Lady fish	*Elops saurus*	++	+	-	-	-	-	++	+
Mullet	*Agonostomus telfairii*	++	+	-	-	-	-	++	+
Pink snapper	*Pagrus auratus*	++	+	-	-	-	-	++	+
Sand perch	*Parapercis millepunctata*	++	+	-	-	-	-	++	+
Sockeye salmon	*Oncorhynchus nerka*	+	++	-	-	-	-	+	++
White grunt	*Haemulon plumieri*	++	++	-	-	-	-	++	++
Speckled trout	*Cynoscion nebulosus*	++	++	-	-	-	-	++	++
Tra	*Pangasius hypothalamus*	+	+	-	-	-	-	+	+

+++: Very strong positive (O.D 415nm higher than 1 or dark purple)
++: Strong positive (O.D 415nm 0.5-0.999 or purple)
+: Weak positive (O.D 415nm 0.2-0.499 or week purple)
+/-: O.D 415nm 0.15 to 0.199 or very week purple stronger color than negative control
-: negative O.D 415 nm lower than 0.15 or no color as negative control
iELISA was loaded 2 ug/100 uL; Dot blog was loaded 1ug/5uL

Table 8 Immunoreactivity of individual MAbs 8F5, 2G9, 2F3 and three pooled MAbs against selected fish (raw) using indirect ELISA and dot blot

Market name	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3		MAbs (8F5 +2F3 +2G9) 1:1:1	
		iELISA 1:20	Dot blot 1:10	iELISA 1:3	Dot blot 1:5	iELISA 1:3	Dot blot 1:5	iELISA	Dot blot
Yellow fin tuna	*Thunnus albacares*	-	-	-	-	-	-	-	-
Blue fin tuna	*Thunnus orientalis*	-	-	-	-	-	-	-	-
Albacore	*Thunnus alalunga*	-	-	-	-	-	-	-	-
Escolar	*Lepidocybium flavobrunneum*	-	-	-	-	-	-	-	-
Wahoo	*Acanthocybium solandri*	-	-	-	-	-	-	-	-
Swordfish	*Xiphias gladius*	-	-	-	-	-	-	-	-
Amberjack	*Seriola dumerili*	-	-	-	-	-	-	-	-
Mahi-mahi	*Coryphaena hippurus*	-	-	-	-	-	-	-	-
Spanish markerel	*Scomberomorus maculatus*	-	-	-	-	-	-	-	-
California basa	*Stereolepis gigas*	-	-	-	-	-	-	-	-

Table 8 – continued

Market name	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3		MAbs (8F5 +2F3 +2G9) 1:1:1	
		iELISA 1:20	Dot blot 1:10	iELISA 1:3	Dot blot 1:5	iELISA 1:3	Dot blot 1:5	iELISA	Dot blot
Red snapper	*Lutjanus campechanus*	-	-	-	-	-	-	-	-
Wild salmon	*Oncorhynchus tshawytscha*	-	-	-	-	-	-	-	-
Tilapia	*Oreochromis mossambicus*	-	-	-	-	-	-	-	-
Cod	*Gadus macrocephalus*	-	-	-	-	-	-	-	-
Seawolf	*Anarhichas lupus*	-	-	-	-	-	-	-	-
Pollock	*Pollachius virens*	-	-	-	-	-	-	-	-
Catfish	*Ictalurus punctatus*	-	-	-	-	-	-	-	-
Whiting	*Merlangius merlangus*	-	-	-	-	-	-	-	-
Basa	*Pangasius bocourti*	-	-	-	-	-	-	-	-

Table 8– continued

Market name	Scientific name	MAb 8F5		MAb 2G9		MAb 2F3		MAbs (8F5 +2F3 +2G9) 1:1:1	
		iELISA 1:20	Dot blot 1:10	iELISA 1:3	Dot blot 1:5	iELISA 1:3	Dot blot 1:5	iELISA	Dot blot
Cubera snapper	*Lutjanus cyanopterus*	-	-	-	-	-	-	-	-
Lady fish	*Elops saurus*	-	-	-	-	-	-	-	-
Mullet	*Agonostomus telfairii*	-	-	-	-	-	-	-	-
Pink snapper	*Pagrus auratus*	-	-	-	-	-	-	-	-
Sand perch	*Parapercis millepunctata*	-	-	-	-	-	-	-	-
Sockeye salmon	*Oncorhynchus nerka*	-	-	-	-	-	-	-	-
White grunt	*Haemulon plumieri*	-	-	-	-	-	-	-	-
Speckled trout	*Cynoscion nebulosus*	-	-	-	-	-	-	-	-
Tra	*Pangasius hypothalamus*	-	-	-	-	-	-	-	-

+++: Very strong positive (O.D 415nm higher than 1 or dark purple)
++: Strong positive (O.D 415nm 0.5-0.999 or purple)
+: Weak positive (O.D 415nm 0.2-0.499 or week purple)
+/-: O.D 415nm 0.15 to 0.199 or very week purple stronger color than negative control
-: negative O.D 415 nm lower than 0.15 or no color as negative control
iELISA was loaded 2 ug/100 uL; Dot blog was loaded 1ug/5uL

Table 9 Intra-assay variability of iELISA

Samples	OD1	OD2	Mean (μ)	Standard deviation (SD)	%CV	Intra-assay variability (%CV)
Yellow fin tuna	2.397	2.385	2.391	0.008485	0.3549	0.15-0.80
Red snapper	2.18	2.203	2.1915	0.016263	0.7421	
Swordfish	2.22	2.24	2.23	0.014142	0.6342	
Blue fin tuna	2.28	2.289	2.2845	0.006364	0.2786	
Albacore	2.456	2.483	2.4695	0.019092	0.7731	
Escolar	2.351	2.378	2.3645	0.019092	0.8074	
Wahoo	2.346	2.341	2.3435	0.00354	0.1509	

OD: Absorbance at 415 nm; CV: Covariance of variation, %CV=SD/μ×100

Table 10 Inter-assay variability of iELISA

Assay	Samples	OD1	OD2	Mean (μ)	Standard deviation (SD)	%CV	Mean %CV	Inter-assay variability
Assay 1	Yellow fin tuna	2.357	2.346	2.3515	0.00778	0.330853	0.435031	0.44 to 0.50
	Red snapper	1.943	1.968	1.9555	0.01768	0.904117		
	Swordfish	2.301	2.323	2.312	0.015556	0.672837		
	Blue fin tuna	2.109	2.1	2.1045	0.00636396	0.302398		
	Albacore	2.335	2.346	2.3405	0.00778	0.332408		
	Escolar	2.307	2.301	2.304	0.00424	0.184028		
	Wahoo	2.447	2.436	2.4415	0.007778	0.318575		
Assay 2	Yellow fin tuna	2.345	2.342	2.3435	0.00212	0.090463	0.435756	
	Red snapper	2.106	2.121	2.1135	0.010607	0.501869		
	Swordfish	2.22	2.24	2.23	0.014142	0.63417		
	Blue fin tuna	2.341	2.326	2.3335	0.01061	0.454682		
	Albacore	2.253	2.234	2.2435	0.01344	0.599064		
	Escolar	2.369	2.355	2.362	0.0099	0.419136		
	Wahoo	2.424	2.412	2.418	0.008485	0.35091		
Assay 3	Yellow fin tuna	2.36	2.355	2.3575	0.00354	0.150159	0.502676	
	Red snapper	2.18	2.203	2.1915	0.016263	0.742094		
	Swordfish	2.22	2.24	2.23	0.014142	0.63417		
	Blue fin tuna	2.28	2.289	2.2845	0.006364	0.278573		
	Albacore	2.456	2.483	2.4695	0.019092	0.773112		
	Escolar	2.351	2.378	2.3645	0.019092	0.807443		
	Wahoo	2.123	2.127	2.125	0.00283	0.133176		

OD: Absorbance at 415 nm; CV: Covariance of variation, %CV=SD/μ×100

Table 11 Sensitivity of assays

Positive samples	**Number of positive samples tested(B)**	**Number of positive results detected as positive (A)**	**Number of positive results detected as negative**	**Portion of positive samples identified correctly as positive**	**Sensitivity**
dot blot assay	240	238	2	238/240	99.2%
iELISA	282	280	2	230/240	99.3%
Pooled MAb-ELISA	32	32	0	32/32	100.0%
Pooled MAb-dot blot	32	32	0	32/32	100.0%
sensitivity= [A/B] ×100=[582/(582+4)] ×100=99.3%					

Table 12 Specificity of assays

Negative samples	**Number of negative samples tested(D)**	**Number of negative results detected as negtive (C)**	**Number of negative results detected as positive**	**Portion of negative samples identified correctly as negative**	**Specificity**
dot blot assay	300	300	0	300/300	100.0%
iELISA	300	300	0	300/300	100.0%
Pooled MAb-ELISA	66	66	0	66/66	100.0%
Pooled MAb-dotblot	66	66	0	66/66	100.0%
Specifity=[C/D] ×100=[732/(732+0)] ×100=100%					

Table 13 Overall accuracy of assays

Number of negative samples tested	732
Number of negative samples tested correctly as negative	732
Number of positive samples tested	586
Number of positive samples tested correctly as positive	582
Total number of negative and positive samples tested (F)	1318
Number of positive and negative samples correctly identified (E)	1314
Overall accuracy=E/F ×100= 1314/1318×100=99.7%	

APPENDIX B

FIGURES

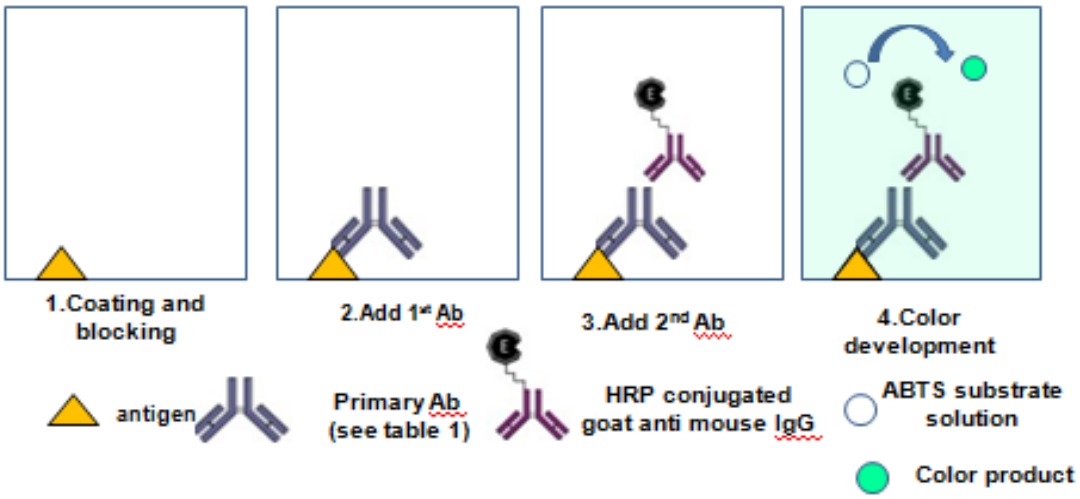


Figure 1. Schematic representation of indirect non-competitive ELISA (iELISA)

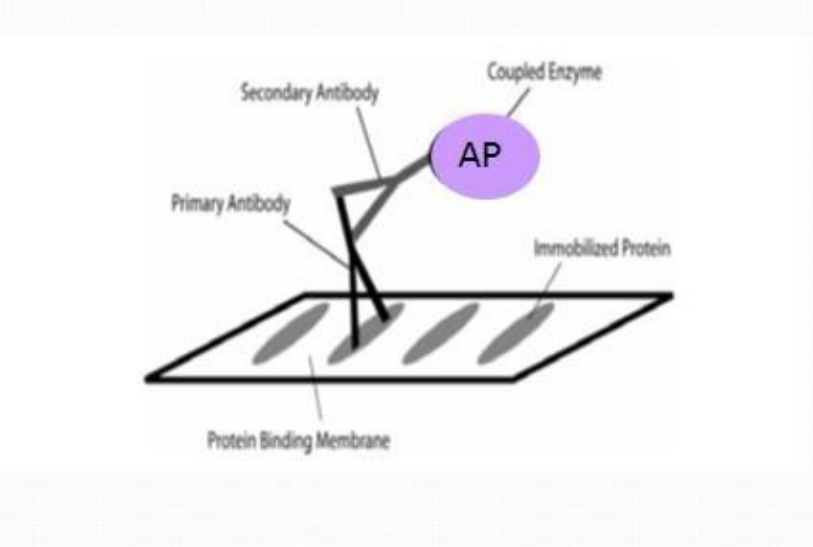
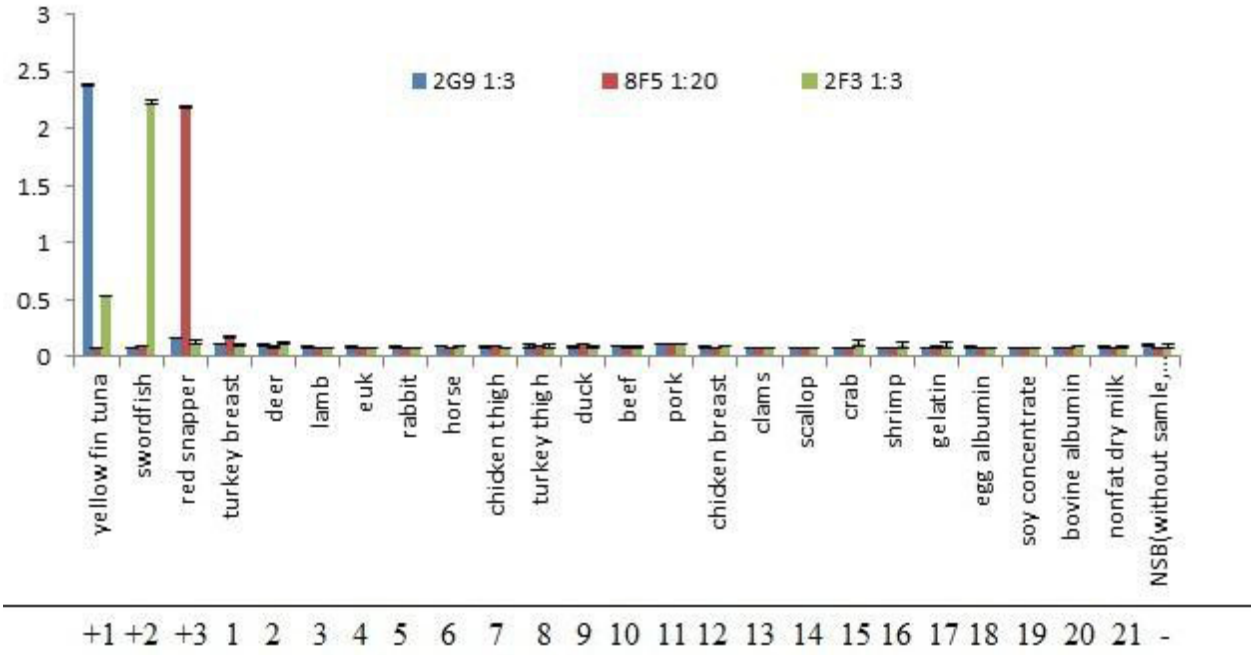
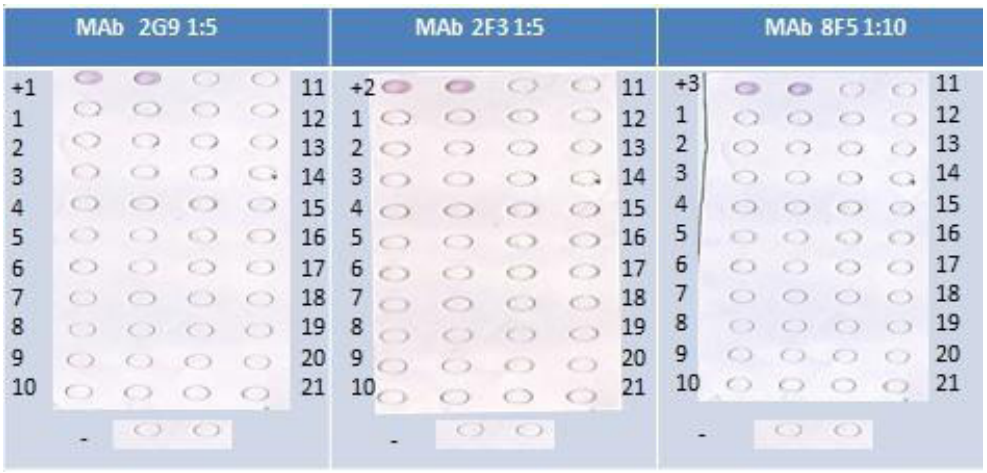


Figure 2. Schematic representation of dot blot



(A)



(B)

Fig 3 Immunoreactivity of MAb 2G9, 8F5 and 2F3 to non-fish samples using iELISA(A) and dot blot(B). iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot).

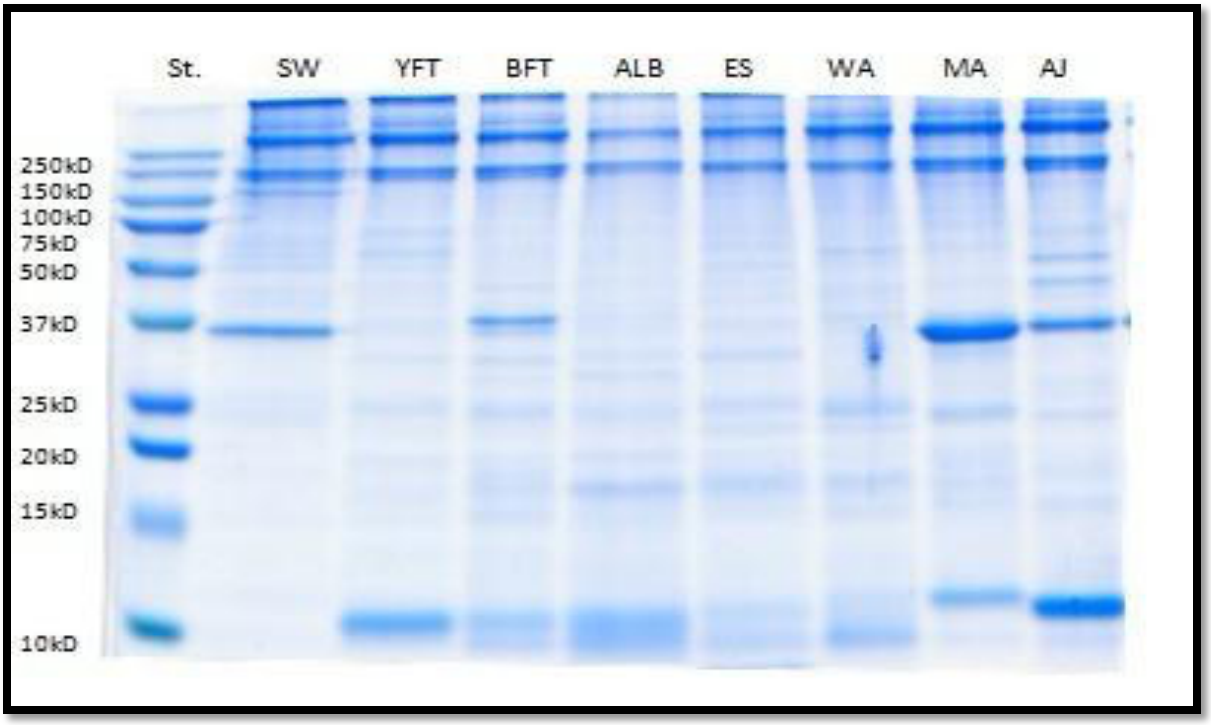


Fig 4 SDS-PAGE protein profile of extracts from 9 species of cooked fish. Samples of extracted soluble protein were loaded 3ug/lane to 12% separating gel and 5% stacking gel. The results show that substantial amounts of thermal-stable proteins are present after cooking (100^{o}C, 8 minutes). St.—Protein Standard; SW— Swordfish; YFT—Yellow fin tuna; BFT—Blue fin tuna; ALB—Albacore (White tuna); ES— Escolar; WA—Wahoo; MA—Mahi-mahi; AJ—Amberjack.

Fig 5 Antigenic protein banding patterns of cooked fish extracts determined by western blot using MAb 8F5(supernatant). MAb 8F5 was diluted 1:10 in 1% BSA-TBST. Samples of extracted soluble protein (3ug in 15 uL per lane) were loaded alongside the Precision Plus protein kaleidoscope standard. Bands correspond to molecular weights of bio-engineered proteins. Goat Anti-Mouse Ig G (H+L)-AP conjugate, diluted 1: 3000 in 10 mL antibody buffer (1% w/v BSA in TBST) was used as the secondary antibody. MAb 8F5 recognized the 36kD protein in mahi-mahi (MA), amberjack (AJ) (shown here) and also all other 57 common fish species tested before (Table 1 and 2) except swordfish (SW), yellow fin tuna (YFT), blue fin tuna (BFT), albacore (ALB), escolar (ES) and wahoo (WA).

Fig 6 Antigenic protein banding patterns of cooked fish extracts determined by western blot using MAb 2G9 (supernatant). MAb 2G9 was diluted 1:3 in 1% BSA-TBST. Samples of extracted soluble protein (3ug in 15 uL per lane) were loaded alongside the Precision Plus protein kaleidoscope standard. Bands correspond to molecular weights of bio-engineered proteins. Goat Anti-Mouse Ig G (H+L)-AP conjugate, diluted 1: 3000 in 10 mL antibody buffer(1% w/v BSA in TBST) was used as the secondary antibody. MAb 2G9 recognized a 25kD antigenic protein in yellow fin tuna (YFT), blue fin tuna (BFT), albacore (ALB), escolar (ES) wahoo (WA), and mahi-mahi (MA).

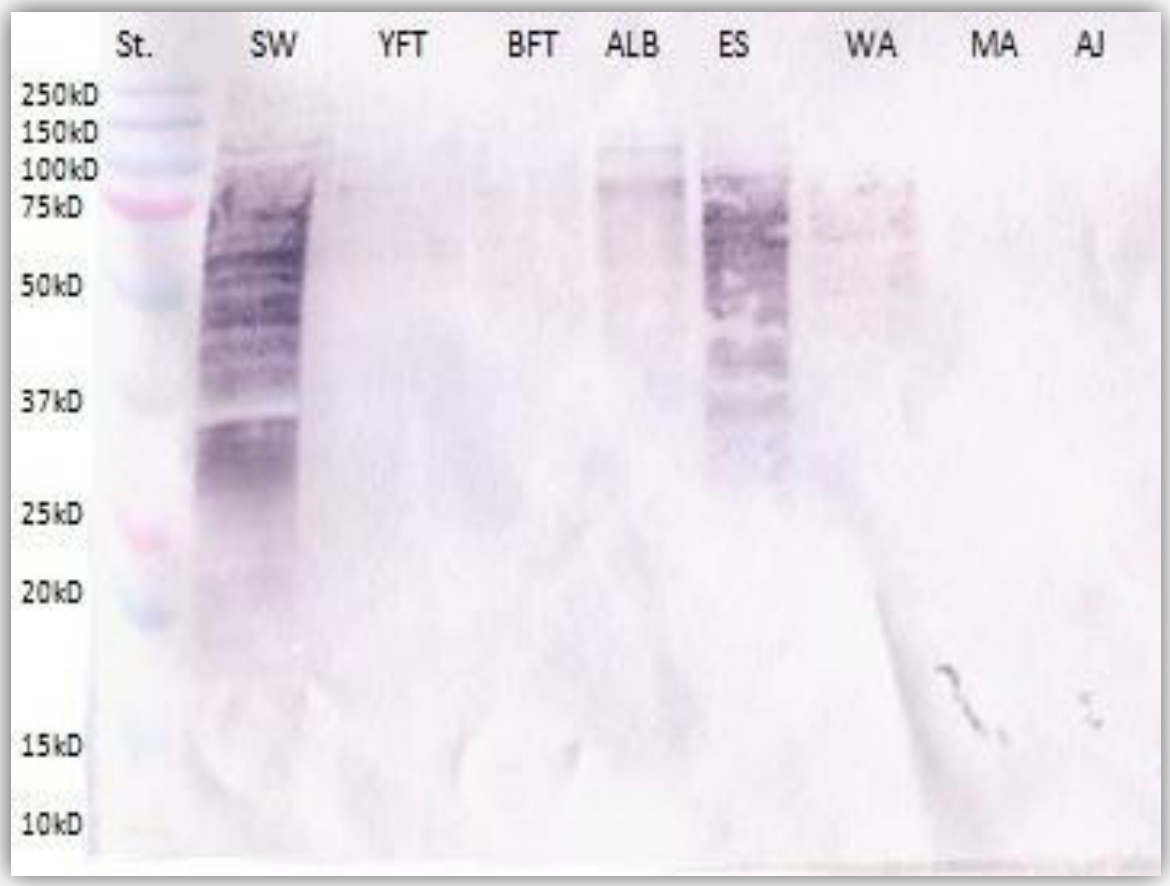
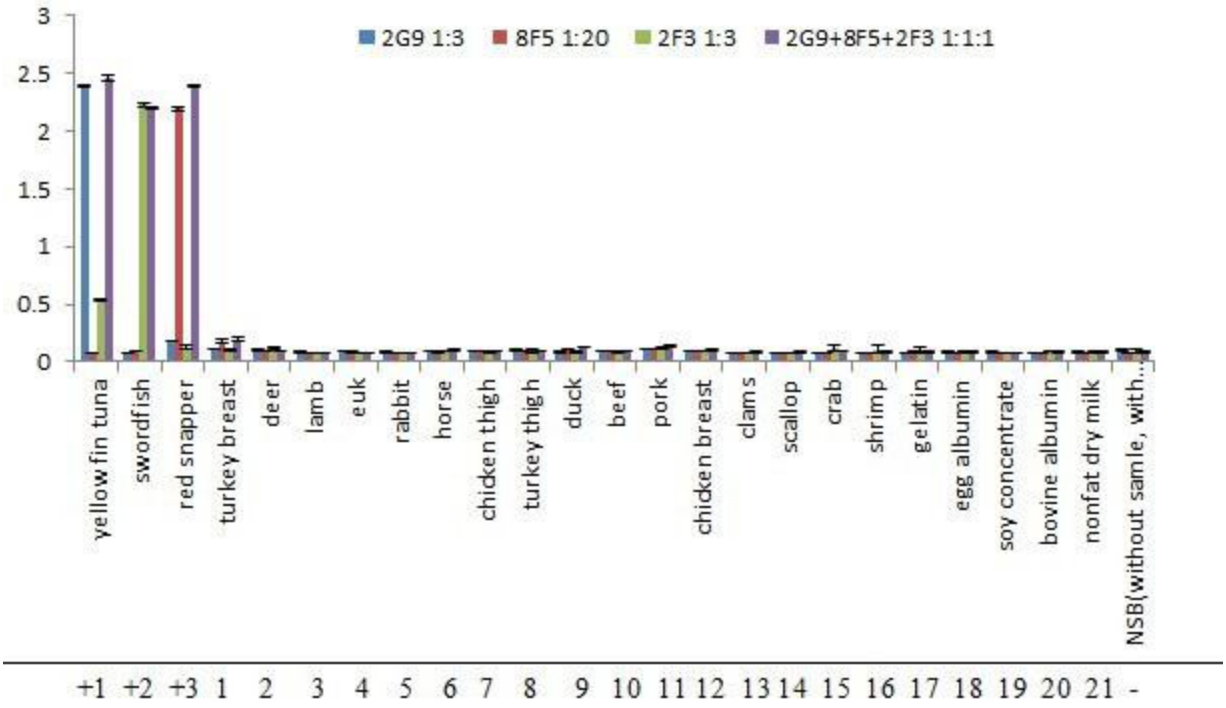
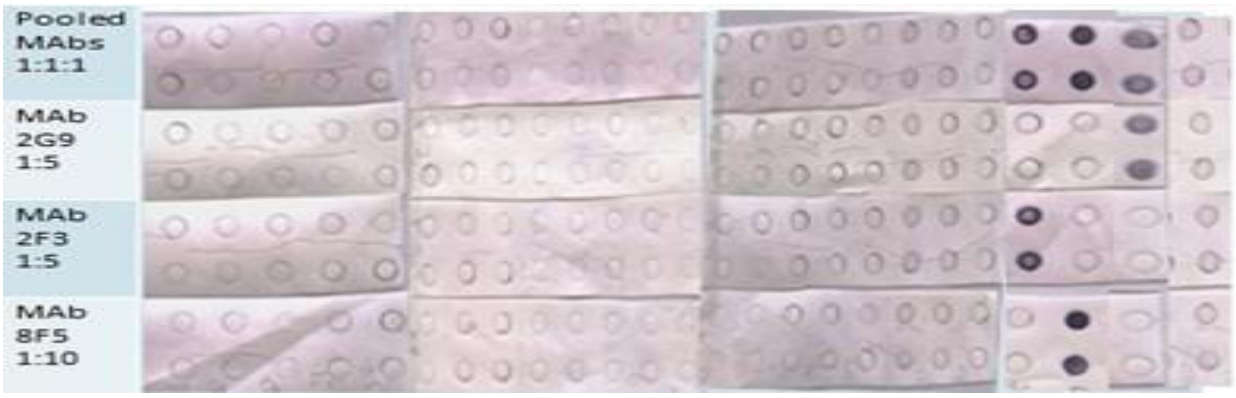


Fig 7 Antigenic protein banding patterns of cooked fish extracts determined by western blot using MAb 2F3 (supernatant). MAb 2F3 was diluted 1:3 in 1% BSA-TBST. Samples of extracted soluble protein (3ug in 15 uL per lane) were loaded alongside the Precision Plus protein kaleidoscope standard. Bands correspond to molecular weights of bio-engineered proteins. Goat Anti-Mouse Ig G (H+L)-AP conjugate, diluted 1: 3000 in 10 mL antibody buffer(1% w/v BSA in TBST) was used as the secondary antibody. MAb 2F3 recognized multiple bands (30-150kD) in swordfish (SW), escolar (ES), wahoo (WA), yellow fin tuna (YFT), blue fin tuna (BFT) and albacore (ALB).

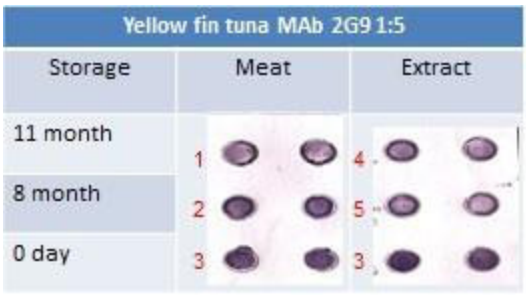


(A)

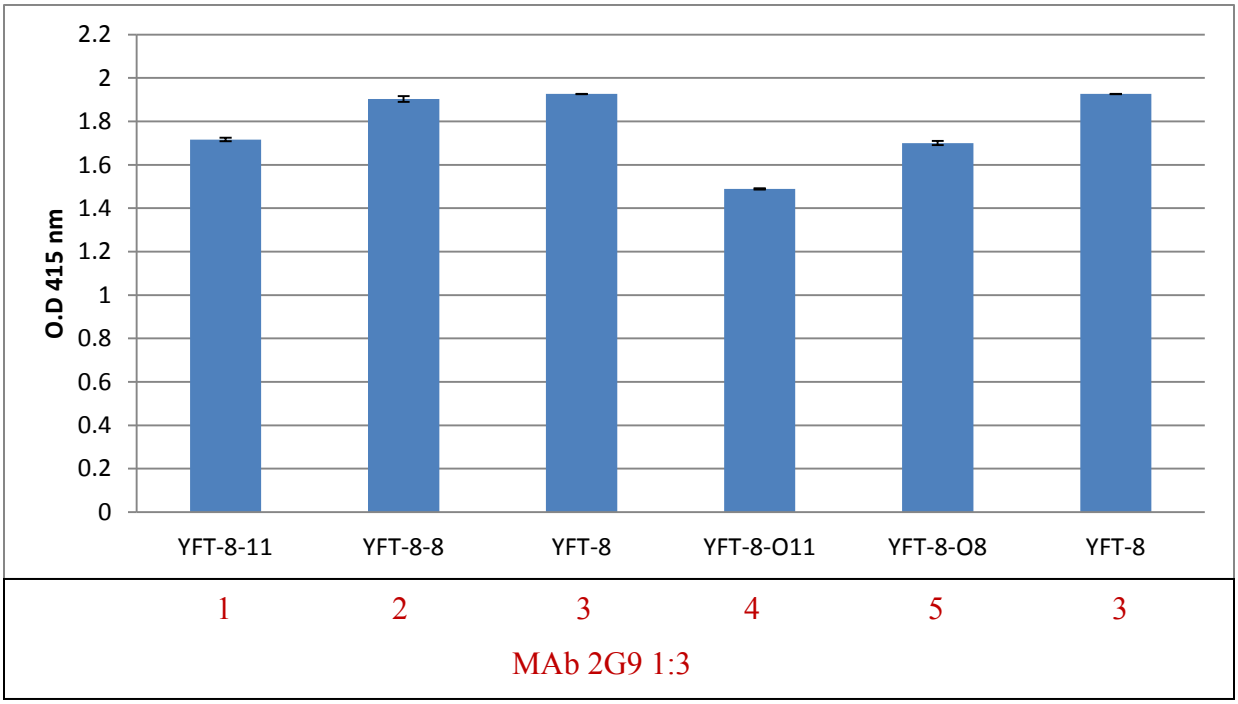


(B)

Fig 8 Immunoreactivity of individual MAbs 2G9, 8F5 and 2F3 pooled to non-fish samples using iELISA (A) and dot blot (B). iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot).



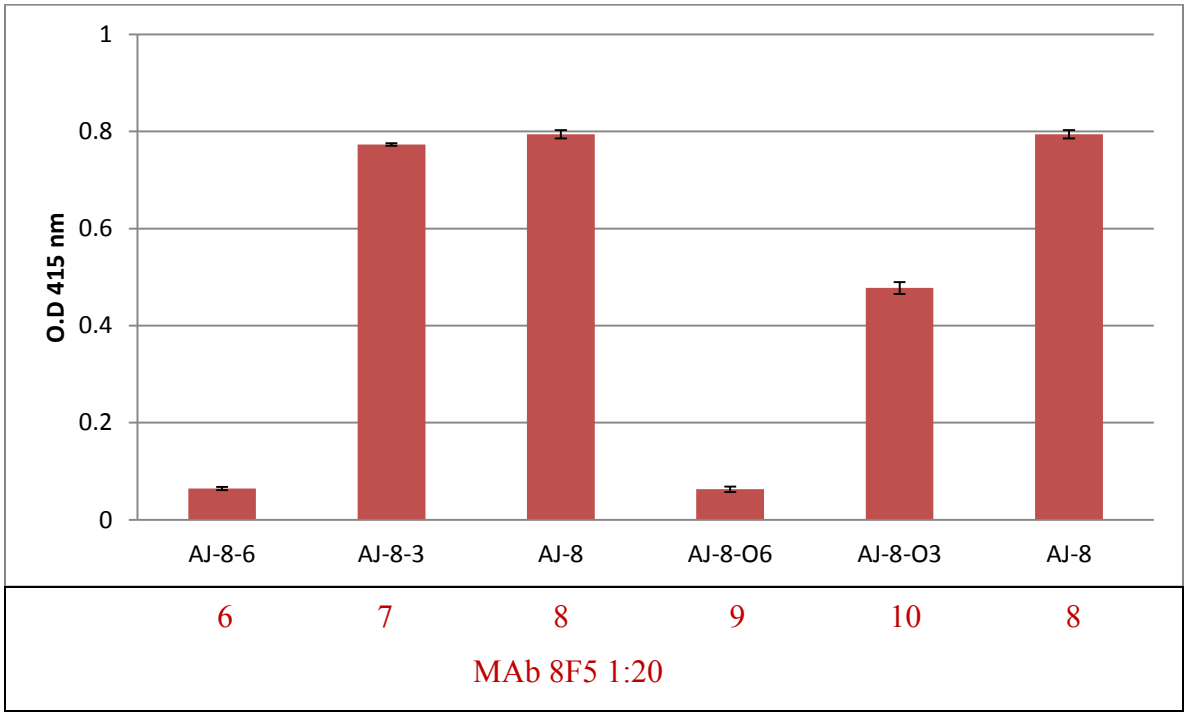
(A)



(B)

Fig 9 Immunoreactivity of MAb 2G9 against yellow fin tuna meat and extract of different storage (meat storage and extract storage) length using dot-blot and indirect ELISA. iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot).

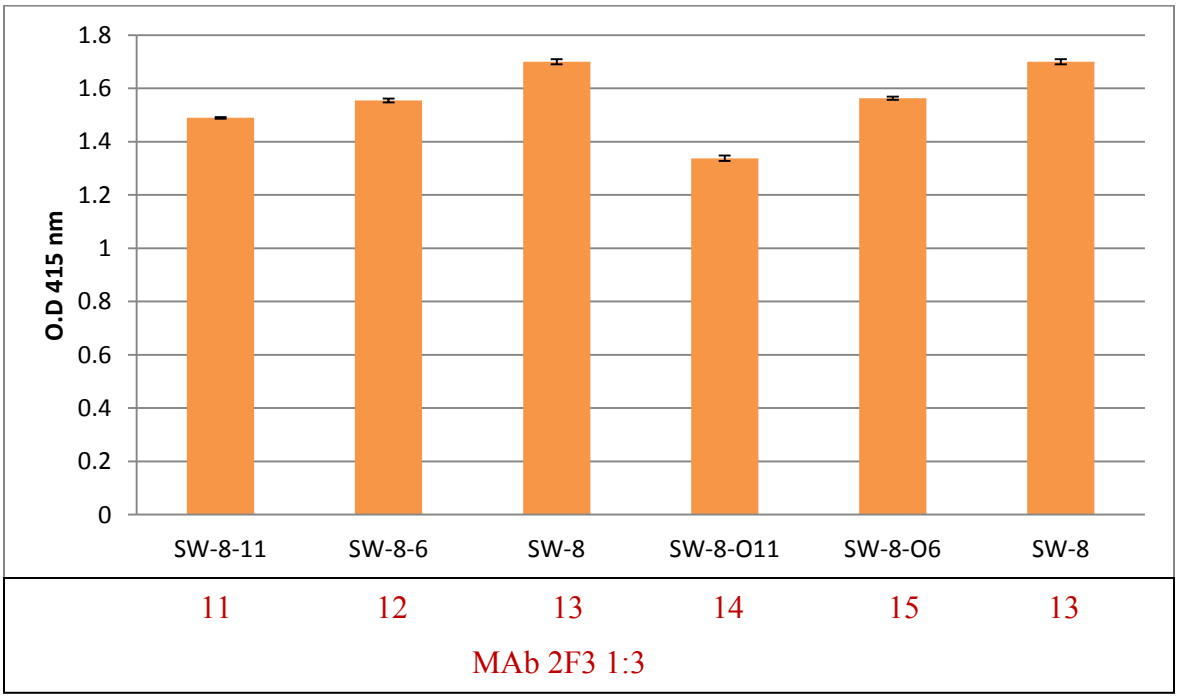
(A)



(B)

Fig 10 Immunoreactivity of MAb 8F5 against amberjack meat and extract of different storage length using dot-blot (A) and iELISA (B). iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot).

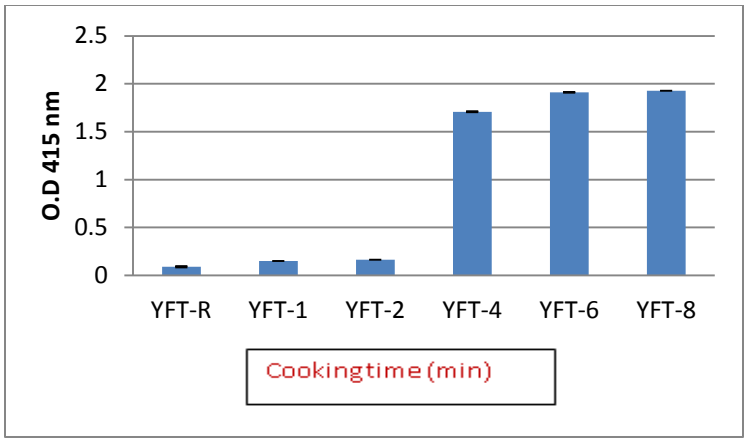
(A)



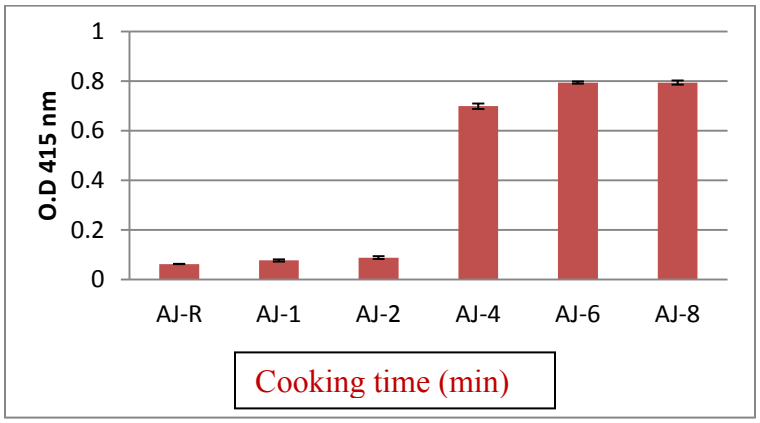
(B)

Fig 11 Immunoreactivity of MAb 2F3 against swordfsih meat and extract of different storage length using dot-blot (A) and iELISA (B). iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot).

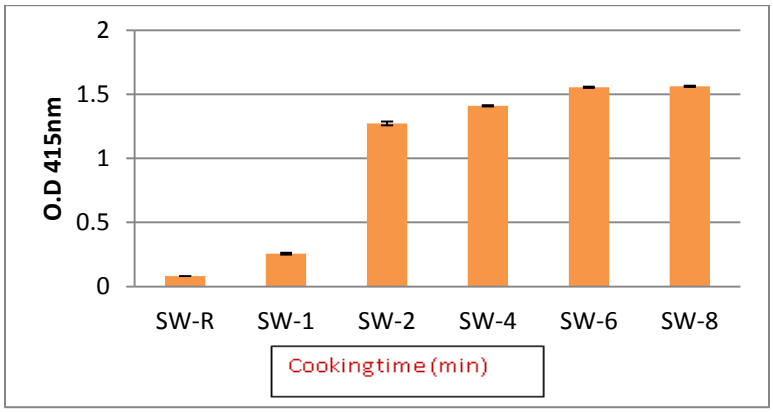
Fig 12 Immunoreactivity of MAbs 2G9, 8F5 and 2F3 against yellow fin tuna, amberjack and swordfish of different cooking length using dot-blot. Protein loaded on 1ug/5ul.



(A)

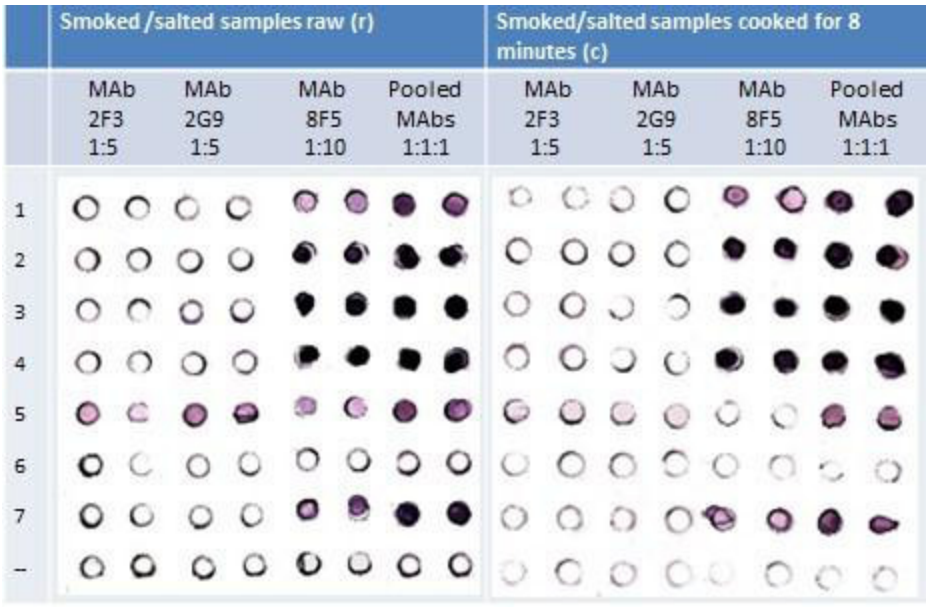


(B)

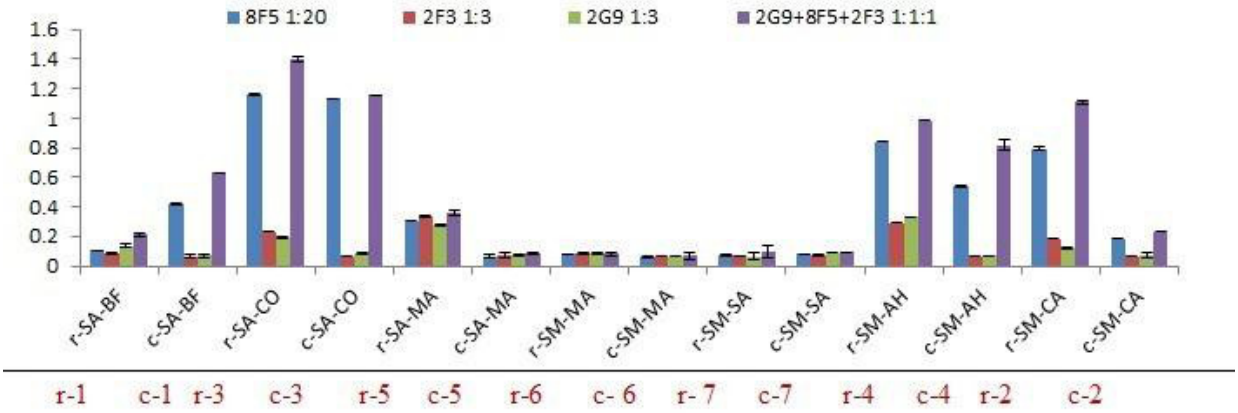


(C)

Fig 13 Immunoreactivity of MAbs 2G9 (1:3), 8F5(1:20) and 2F3 (1:3) against yellow fin tuna (A), amberjack (B) and swordfish (C) of different cooking length using iELISA. iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot).



(A)



(B)

Fig 14 Immunoreactivity of MAbs (8F5, 2F3, 2G9 and pooling of three) to raw and cooked processed fish using iELISA(A) and dot blot (B). iELISA results are expressed as the mean of O.D 415 nm± SD. Protein loaded on each well was 2 ug/100ul (iELISA), 1ug/5ul (dot blot). 1-salted butter fish, 2-smoked carp, 3-salted cod, 4-smoked Atlantic herring, 5-salted markerl, 6-smoked mackerel, 7-smoked salmon, -negtive control.

APPENDIX C

ABBREVIATION LIST

2-ME-2-mercaptoethanol

Ab-antibody

ABTS-2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

ANOVA- analysis of variance

Anti-IgG-HRP-goat anti-mouse IgG (Fc specific) horseradish peroxidase conjugate

AP-alkaline phosphatase

BSA-bovine serum albumin

BSE-bovine spongiform encephalopathy

CV-coefficient of variation

ELISA-enzyme-linked immunosorbent assay

FALCPA-Food Allergen Labeling & Consumer Protection Act

FDA-Food and Drug Administration

h-hour

H_2O_2-hydrogen peroxide

HRP-horseradish peroxidase

iELISA- indirect non-competitive enzyme-linked immunosorbent assay

IgG-immunoglobulin G

LOD-limit of detection

MAb-monoclonal antibody

Min-minute

MP-myofibrillar protein

MSG-monosodium glutamate

MTGase-microbial transglutaminase

MW-molecular weight

Na_2CO_3-sodium carbonate

NaCl-sodium chloride

$NaHCO_3$-sodium bicarbonate

Na_2HPO_4-sodium phosphate dibasic anhydrous

NaH_2PO_4-sodium phosphate monobasic anhydrous

NaOH-sodium hydroxide

NFDM-nonfat dry milk

OD-optical density

PAb-polyclonal antibody

PBS-10 mM phosphate buffered saline (pH 7.2)

PBST-PBS containing 0.05% Tween 20 (ml/ml, pH 7.2)

Ppm-parts per million (μg/ml)

SDS-sodium dodecyl sulfate

SDS-PAGE-sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS-tris-buffered saline

TBST-20 mM TBS containing 0.05% Tween 20 (ml/ml, pH 7.5)

TEMED-N, N, N', N'-tetra-methyl-ethylenediamine

TGase-transglutaminases

Tris-tris(hydroxymethyl)aminomethane

APPENDIX D

BUDGET

Salary	
Yaozhou Zhu, Master candidate	
1 year GRA@ 26,000.00 per year)	$ 26,000.00
Fringe benefits @ 1.75%	$ 525.00
Total salary	**$ 26,525.00**
Supplies	
Chemicals and laboratory ware	$ 2,000.00
Antibodies reagents	$ 3,000.00
Samples and other supplies	$ 500.00
Stationery	$ 100.00
Total supplies	**$5,600.00**
Travel	
Travel to professional meetings (IFT)	$ 1,200.00
Total travel cost	**$ 1,200.00**
Publications	
Manuscript (@ $1,000.00 per manuscript)	$ 1,000.00
Total publication costs	**$ 1,000.00**
Direct cost	
Salary	$26,525.00
Supplies	$ 5,600.00
Travel cost	$ 1,200.00
Publications	$ 1,000.00
Total direct cost	**$ 34,325.00**
Indirect cost (25% of total direct cost)	**$ 8,581.25**
TOTAL PROJECT COST	**$ 42,906.25**

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BIOGRAPHICAL SKETCH

Yaozhou (Sophie) Zhu

Educational Background

08/2010-present	Florida State University	Tallahassee, FL, USA
	M.S in Food Science and Nutrition	
	Major Professor: Dr.Yunhwa Peggy Hsieh	
08/2006-07/2010	Shanghai Ocean University	Shanghai, China
	B.S in Food Science and Engineering	
	Fudan University	Shanghai, China
	Minor in Finance	

Honors and Awards

09/2012 *Golden Key Honor Society*, Florida State University Chapter

07/2012 *Travel Grant*, Congress of Graduate Student (COGS), Florida State University

06/2012 *3rd place*, Student Paper Competition, Institute of Food Technologists(IFT) Annual Meeting 2012

04/2012 *Betty M. Watts Memorial Scholarship*, Nutrition, Food and Exercise Sciences Department, Florida State University

07/2011 *Florida China Linkage scholarship*, 9 credits tuition waiver

07/2010 *Honoring Graduates*, awarded by Shanghai Ocean University

10/2009 *People's Scholarship,* awarded by Shanghai Ocean University

09/2009 *"Outstanding Student Role Models",* awarded by Shanghai Ocean University

10/2008 *Excellent Student,* awarded by awarded by Shanghai Ocean University

10/2008 *"Excellent League Member",* awarded by Shanghai Ocean University

06/2008 *Second Prize* in debate competition, awarded by Shanghai Ocean

	University
03/2008	*People's Scholarship,* awarded by Shanghai Ocean University
10/2007	*People's Scholarship,* awarded by Shanghai Ocean University
03/2007	*People's Scholarship,* awarded by Shanghai Ocean University
01/2007	*"Excellent League Cadre",* awarded by Shanghai Ocean University
01/2007	*"Excellent Award"* in speech contest, awarded by Shanghai Ocean University

Research interests

- Development of immunoassay for rapid detection for seafood safety
- Physical and chemical properties of seafood during processing

Research Experience

08/2010-now — Assistant in Department of Nutrition, Food and Exercise Sciences, Florida State University

- Detection of fish species substitution using immunoassay

07/2009-04/2010 — Assistant in Marine Products Processing Laboratory, Shanghai Ocean University

- Studied the flavor substance of different parts of grass carp, and compared the flavor substance of different parts of wild grass carp to those raised by humans in different seasons; Studied the volatile flavor substance of different seaweeds; Studied the aroma components of surimi in different rinsing conditions

Instruments: GC-MS, SPME, E-NOSE

04/2009-08/2010 — Assistant in Food Nutrition and Safety Lab, Shanghai Ocean University

Subject: Volatile Components and Sensory Characteristics and Consumer Liking of Commercial Brand Oyster Sauces

11/2008-03/2009

- Investigated the food in the canteen where 90% students have dinner and their health condition got in the physical examination
- Investigated the diet of randomly selected 500 students and their weights in long term
- Calculated the normal distribution of the students' nutrition in our university and in the whole country through the mathematics methods according to the feedback information, then made different recipes according the nutrition conditions, then made into a computer program
- The students in our university can find their nutrition condition and recommendatory recipes according to their weights

04/2008-06/2008 Team Leader in the program of California Almond Food Research and Development Competition

- Designed the research and development of the California Almond, and made the marketing plan

Presentations with Published Abstracts

Zhu Y and Hsieh Y-H.P. Pooling of MAbs for rapid detection of commercially important fin fish. Poster session and peer reviewed abstract published. Presentation Number 2012 Annual Meetings of Institute of Food Technologists. June 25-28. Las Vegas, NV

Zhu Y and Hsieh, Y-H.P. Immunochemical detection of common species of fish using pooled monoclonal antibodies. Feb, 2012. Poster session. Research creativity day, Nutrition, Food and Exercise Sciences Department, The Florida State University.

Publications

Shi W, Wang X, Tao N, Zhu Y and Liu Y. 2011. Volatile components of wild and cultured grass carps. Jiangsu Journal of Agricultural Sciences, 27(1): 1-5.

Shi W, Wang Z, Zhu Y, Zhang Z. and Wang X. 2009. Research on changes of the volatile compounds of grass carp surimi in the washing process. Science and Technology of Food Industry, 12:116-123

Nguyen T, Wang X and Zhu Y. 2009. Volatile components and sensory characteristics and consumer liking of commercial brand oyster sauces. SPISE-Summer Program in Sensory Evaluation, 8:123-139

Teaching Experience

Fall 2012	Teaching assistant Food Lab
	Florida State University
Spring 2008	Teaching assistant Sensory Evaluation I
	Shanghai Ocean University

School Activities & Leaderships

04/2008-09/2009 *Vice President* of GO-GO CAUMP-ORIGENTERING, Shanghai Ocean University

- Organized students to do the orienteering and painted the map of

orienteering

10/2006-06/2008 *Volunteer*, Shanghai Geracomium in Nanhui District

- Organized students to talk and play chess with seniors

10/2006-06/2007 *Member*, Shanghai Science and Technology Museum

- Taught volunteers about the health and nutrition knowledge, and instructed and assisted them to play some games

10/2006-06/2007 *Assistant*, Association for spectacular fish fans (one of the 20 famous associations in Shanghai)

- Be responsible for some accounting work

Work Experience

Intern, International Funds for China's Environment of D.C, U.S

08/2009-12/2009

Shanghai, China

- Assistant work for translation of business documents
- Assistant work for the activity organization

08/2008-10/2008

Assistant in Research and Development Department, Jiansimao Food Co., Ltd. of Changsha, Hunan

Changsha, China

- Researched and developed spicy small fish
- Researched and developed bean cured with chicken soup

07/2007-09/2007

Assistant, Shanghai Tianyu Law Firm

Shanghai, China

- Searched and explained professional knowledge and the hidden trouble about food like illegal additive in the lawsuit of food company
- Designed recipes for staff
- Some assistant work

Professional memberships

Institute of Food Technologist

IFT Florida Section