Identification and Quantification of Pomfret allergens

Dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

by

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May 2022

DECLARATION

I hereby confirm that the work proclaimed in the M.Tech thesis entitled "**Identification and Quantification of Pomfret allergens**" submitted at Jaypee University of Information Technology, Waknaghat is a credible record of my work carried out under the supervision of Dr. Garlapati Vijay Kumar. The results embodied in this thesis have not been submitted to any other university or institute for the award of any degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to affirm that the work entitled "**Identification and Quantification of Pomfret allergens**" submitted by Priyanjalee Bhattacharjee (202551) in partial fulfilment of the requirement for the award of Master's of Technology Degree in Biotechnology of Jaypee University of Information Technology, Waknaghat is an authentic work carried out by them under by supervision and guidance. The matter embodied in this report is original and has not been submitted for the award of any other degree.

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ACKNOWLEDGEMENTS

We would like to thank God for guiding us throughout our academic journey and to acknowledge our project supervisor, Dr, Garlapati Vijay Kumar, for his undying support, priceless motivation and guidance throughout the project duration. Moreover, we extend our sincere gratitude towards the lecturers and the non-teaching workforce of the Department of Biotechnology and Bioinformatics towards their donation towards the success of this work.

The responsibility our associates played during the entire phase should not go unstated. Thank you all for your decent hold up and support. We deeply honored and obliged to you all.

As to our family, we appreciate the sustain help you have given us all over our academic expedition. This seek has not been easy but you have always gravely stood by our side.

Thank you.

(Priyanjalee Bhattacharjee)

ABSTRACT

Food-actuated sensitivities are viewed as a significant issue of general wellbeing with extraordinary effect in the personal satisfaction of the sharpened/hypersensitive people. As exceptionally eaten food sources, fish and shellfish address a significant wellspring of proteins for the overall public. Despite their efficient and wholesome significance, these food sources are known to instigate touchiness responses in sharpened/hypersensitive people. Up to this point, fish's parvalbumins and scavangers & molluscs tropomyosins have been thought about significant allergens in fish sensitivity, being answerable for a large portion of the detailed instances of unfavorable immunological reactions. All the more as of late, different proteins such as myosin light chains, arginine kinases, sarcoplasmic calcium-restricting proteins and troponins have been viewed as significant allergens in fish, shellfish and molluscs. This report centers around the allergens of raw, boiled and fried pomfret, with an outline on the most delegate insightful techniques for their identification and quantification. Though the distinctive evidence and evaluation of a couple of fish allergens have been reported but there is no data related Indian fish, var. pomfret allergens and the effect of cooking conditions of allergenicity. Hence, the present study intended to isolate the allergic proteins of pomfret and its subsequent s studies on allerginicity and quantification by revealing the effect on allerginicity with food processing step.

Keywords: Pomfret; Allergen; Proteins; Skin prick test; Myosin

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CHAPTER 1
Introduction

Fish assumes a significant part in human nourishment and wellbeing, which is thought of as a great wellspring of exceptionally absorbed proteins, nutrients and polyunsaturated unsaturated fats, for example, docosahexaenoic corrosive and eicosapentaenoic corrosive. There are areas of strength for a foundation enumerating the wide variety of clinical benefits connected with the consumption of omega-3 unsaturated fats, such as the prevention of cardiovascular disease and infection, as well as the improvement of glycemic management. Because of the extensive medical benefits of fish and shellfish, their consumption has been rapidly expanding over the world. The growing interest in fish confirmation might be considered a refreshing benefit for the bulk of the world's populations. However, the use of foods containing undeclared fish can result in major health difficulties (for example, basic immunological responses, hypersensitivity) for a small but critical group of food-unfavorably vulnerable people as a result of coincidental exposure to the guilty meal. In recent years, more cases of fish and shellfish sensitivities have been reported, and they are now considered a growing public health concern. Rather than open food challenges (OFC) or twofold outwardly hindered counterfeit treatment controlled food challenges, the clinical examination of unequivocal food awareness's, such as fish responsiveness, relies on self-declared aftereffects (clinical history), express the true regularity of fish awareness is difficult to spread out either through sIgE-based blood tests or skin prick test (SPT) sensitization tests. The transparent pathways of fish responses include ingestion, internal breath of scents, direct contact (skin), and fume's of processed predefined food sources. The complete avoidance of fish or the administration of a supportive medication (such as antihistaminics,

corticosteroids, or epinephrine) because of an accidental receptiveness to the allergenic meal is the most successful strategy for thwarting a hostile reaction in severely hypersensitive persons. Subsequently, it became basic to work on purchaser's security through an exact food naming framework, to forestall potential life-threatening gambles for sharpened/unfavourably susceptible people. This article aims to provide a broad and energising perspective on fish allergies, counting a concise portrayal of the most agent insightful techniques for their identification and quantification.

The likely reason for IgE-intervened fish excessive touchiness is because of ingestion of fish or internal breath of exhaust during taking care of or cooking of fish. Utilization of fish items could prompt a few unfavorably susceptible side effects, for example skin rash, dermatitis, urticaria, angioedema, looseness of the bowels, spewing, respiratory trouble and, surprisingly, lethal fundamental anaphylactic responses. Various types of heating treatment can modify the formation of some allergens, which are necessary proteins or glycoproteins, resulting in a change in allergenicity. The heat-stable food allergenic proteins found with the eggs, fish, milk, peanut foods whereas heat-labile one's are found with the food stuff of cereals, celery and soybeans. The members of Rosaceae and carrots proteins falls under heat-resistant. Numerous allergens are for the most part impervious to proteolysis or stomach related proteins. Most allergenic food proteins, for food hypersensitive population require broad modification to reduce their allergenicity. Non-allergenic baby equations are delivered for newborn children with pasteurized milk sensitivity by heat degradation and enzymatic polymerization of milk proteins. It was shown that various types of hotness impacted the allergenicity in various ways. The protein of nut remain unaltered while cooking expanded the allergenicity, bubbling or broiling decreased because of its allergenic potential. According to reports, the allergen's IgE-restricting limit has moderately extended on capacity due to the introduction of new proteins. Albeit the impacts of warm handling on eggs, endlessly milk

items have been concentrated widely, a couple have zeroed in on fish proteins. Fish handling like storing and freeze-drying modified some of the significant fish allergens liable for ImmunoglobulinE-intervened fish sensitivity. Fish are generally exposed to some type of warm handling, particularly bubbling or broiling, before utilization. As having little information about heat- sensitive and resistant fish allergies, the present study is intended to assess the effect of heating on pomfret allerginicity.

CHAPTER 2
Review of Literature

Only a few protein families have been identified as immunological triggers in fish, including enolases, aldolases, and parvalbumins, with parvalbumins being the most common. Ca-restricting proteins, which are the second most important type of creature food immune triggers, are found in parvalbumins. Allergens in Fish of now announced as answerable for over 95% of food sensitivities initiated by fish. By and large, the side effects happen 30 minutes after consuming the culpable food and can bring side effects in skin, breathing problem and gut problem including less incessant lethal fundamental reactions like hypersensitivity.

2.1. Parvalbumin

Parvalbumins are small, acidic, and water-soluble proteins with a molecular weight of 10-13 kDa.introducing momentous protection from high temperatures, denaturing specialists and proteolytic movement. They are as a rule partitioned into two transformative heredities of isoforms: the α-parvalbumins, which are for the most part named hypoallergenic, and the -parvalbumins, which contain the majority of ImmunoglobulinE-receptive parvalbumins. Many fish species have a lot of parvalbumins in their white tissue playing out a significant job in the unwinding of tissue strands by limiting the free Ca which are inside the tissue. They are created by two utilitarian spaces, each limiting a Caparticle, and a third quiet area safeguarding the non-polarcenter of the protein. In these proteins, the limiting of Ca is the basic significance to the uprightness of the IgE epitopes' adaption Ca intake is known to cause main changes in these proteins, lowering

parvalbumins' allergenic limit.These sarcoplasmatic proteins are abundant in the habitats of these fish species, such as saltwater fish, whiffs, and wallows. As a result, these species are thought to be more allergenic than dynamic fishes (those with a lot of dark muscle), such as fish, saltwater fish, yellowfin, and skipjack. Resilience to specific fish animal groups can vary greatly among adversely susceptible persons, therefore a sharpened patient has about a half-chance of being cross-receptive to multiple fish animal groupings. This is due to the fact that whereas parvalbumins' auxiliary and tertiary designs are well kept, their amino corrosive groupings (essential structures) can differ dramatically between fish 2 species. Limited information on epitope arrangement of four parvalbumins from various fish species, in particular blue jack, saltwater fish, yellowfin and beef, appear to demonstrate the presence of an exceptionally antigenic area, which may be accountable for the softening of various fish species in unfavourably susceptible people. Current cross-reactivity research highlights the necessity for sharpened/hypersensitive persons to eliminate all types of fish from their diets, even before receiving sensitivity results from SPT, serum-explicit IgE blood tests, or OFC.Because parvalbumins are considered essentially stable proteins, they are often impenetrable to normal physical and chemical processes. The fate of allergenic proteins during food preparation is another important feature of fish sensitivity. At this time, it's unclear the rationale of impacting of parvalbumins through food processing of different types of fishes. The recent studies suggested that foods heating won't find any substantial IgE-restricting thresholds due to the returning to normal confirmation on cooling. Substance processes can lower the IgE-restricting limit of parvalbumins. Proteolysis, which is usually combined with pH changes, is another effective method for reducing allergenicity; however, it may also help to disclose previously hidden epitopes or create new epitopes through accumulation. Since most fish-unfavorably susceptible individuals do not tolerate saltwater fish,

saltwater fish sensitivity is now the greatest all-around thought. Gad c 1, a significant allergen isolated from Baltic cod (Gaduscallarias), is commonly employed as a source of perspective particle in parvalbumin research. Other cod species (Gadusmorhua), normal carp (Cyprinuscarpio), Atlantic salmon (Salmosalar), Thunnusobesus (bigeye fish), *Trachurus japonicas* (Japanese jack mackerel),and European hake possess similar allergens. As of late, the quantity of recognized allergenic proteins accessible at data sets has expanded, working on the foundation of developmental and underlying connections among allergens from unmistakable beginnings. The majority of well-known fish allergens are parvalbumins, which comprise more than 200 passages, albeit different proteins, specifically enolases and aldolases, are likewise characterized as ImmunoglobulinE-receptive in fish species.

2.2.Aldolases and Enolases

When it comes to allergenic proteins in fish, enolasess(50 kDa) and aldolases (40 kDa) were the major allergens with saltwater fish, blue jack, and fish.The two catalysts have natural capacities in metabolic EMP pathway, being associated with the glucose corruption for the development of energy. The biological depiction of enolases appears to show that the proteins are dimeric in nature, while aldolasesshow the profiles for oligomeric. Furthermore, in fish enolases and aldolases, no post-translational changes such as acetylation or ubiquitination have been identified. Cross-reactivity between species was found to be restricted and found that enolases were more cross-responsive than the aldolases. The tri-layered structures of enolases and aldolases were affected with cooking above 90°C for 1-5 mins. indicating that they are heat unstable. Despite the eradication of some conformational allergic epitopes, food handling can result in the development of new straight epitopic areas, potentially increasing allergenicity.Collagen, in addition to parvalbumins,enolases and aldolases have been identified as allergens. In any case, the danger of fish gelatine (collagen)

evoking an unfavourable immunological response in fish-unfavorably predisposed people remains unknown, despite the availability of several reliable in vitro sensitivity assays.

2.3. **Shellfish Allergen**

The phrase "shellfish" refers to a non-ordered assignment that is commonly used in the context of fish consumption. Shellfish and mollusks are included in this gathering, which addresses a large market speciality of marine species with a high commercial premium.Arthropods, which include more than 50,000 live species, are called shellfish (shrimp, prawns, lobster, crawfish and barnacles). Countless scavenger species are eaten raw or after being cooked or handled. Mollusks are classified into three groups: bivalves, gastropods, and cephalopods, and there are over 100,000 different species (mussels, shellfish, abalone and squids). Mollusks are highly valued and eaten food variety everywhere, especially in seaside districts, due to their health benefits and intrinsic taste features. After 2 hours of consumption, touchiness responses to fish are usually prompt (approximately 30 minutes). Late-stage immunological reactions are also possible, especially when adverse effects last for up to 8 hours.Clinical appearances of shellfish sensitivity are basically the same as fish sensitivity, coming about, not just from the ingestion of the culpable food, yet in addition from controlling or breathing in the preparing fumes during food handling. Generally, side effects start in practically no time and may incorporate oral sensitivity disorder and cutaneous (urticaria, angioedema), gastrointestinal (regurgitating, stomach torments) or potentially respiratory side effects. Albeit less incessant, serious and fundamental reactions, for example, anaphylactic shocks may likewise happen upon shellfish utilization.

2.3.Tropomyosins

In shellfish and molluscs, various proteins are known to set off perceptible clinical side effects, albeit most of them belongs to tropomyosins which related to the α-helix looped curl auxiliary construction proteins. Tropomyosins are found in muscle and non-muscle cells, besides, alongside actin and myosin, they mediate in the authoritative course of muscle pressure. Up until this point, a staggering number of allergenic tropomyosins have recently been depicted among shellfish, explicitly in whelk, crab, cross-reactivity in lobster and shellfish is linked to a high degree of amino destructive plan similarity among unquestionable species. The similarity across most shellfish-sensitive people cross-react when they eat different bivalve or mollusk species because tropomyosins levels are so high. Because of the high primary similarity across tropomyosins, it is estimated that 75% of those with current sensitivity to some type of shellfish are at risk of cross-reacting to a second animal variety. Tropomyosins are heated stable proteins with subatomic loads ranging from 34 - 38 kDa in MW which were unfold to a limited extent with warming process and refolded back with the cooling of food. Compound cycles, such as Maillard changes, may increase tropomyosin allergenicity.

2.4.**Arginine Kinase**

As of late, different proteins, for example, Arginine kinases were identified as novel allergens with shellfish and mollusks . Arginine kinase (40 kDa, water-soluble protein) mostly found in myosinogen and serves as a catalyst in the cell's digestion process in case of spineless organisms. Starter gives information on allergic arginine kinases from shellfish. These proteins appear to be sensitive to 40°C -80°C and unfolded which reveals the responsibility of novel secret epitopes in enhance IgE reactivity. This protein unfurl and accordingly decline its immunogenicity completely when the temperature goes above 80°C. The arginine kinase

from molluscs becomes unsound when the temperature goes above 40°C, compared to the arginine kinase from shellfish however writing proposes that its allergenic properties are decreased when the temperature goes low.

2.5.Troponin C proteins and Sarcoplasmic ca-binding

The sarcoplasmic Ca-restricting and troponin C proteins are members of the EF-hand protein superfamily.The sarcoplasmic Ca-restricting proteins are available in the spineless creatures, being viewed as what might be compared to the vertebrate parvalbumins that add to keep up with the calcium inside the vertebrates. They are known as ca-cradles, which are acidic cytosolic proteins with molecular weights ranging from 20 to 22 kDa and four possible EF-hand Ca-restricting sites, only a few of which are practical. Troponin C is a Ca-detector/regulatory protein that controls the Ca-subordinate management of downstream goal proteins.Up to this point, in scavengers, 26 sarcoplasmic Ca-restricting proteins and 5 troponin proteins have been distinguished as allergens, despite the fact that their review is currently at an extremely starter stage.

2.6.Myosin

Myosins have a role in a complex system with a set of proteins i.e., actin, troponin, and tropomyosin which plays an important role in tissue withdrawal. It is made by heavy chains (two) and light chains (four). Each myosin heavy chain is surrounded by a two light chains (20 kDa. Recently, the six myosin light chains have been identified as allergens in scavengers. The light chain allergens of myosin exhibits a high IgE reactivity with shellfish hypersensitive patients serum which indicates the profound allergens related to the tropomyosin allergens. The light chain allergens are impervious to warm as quite often keep up with IgE-restricting limit by handle it at 100°C for 5 mins.

CHAPTER 3

Methodology

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3.1 Preparation of Fish Extracts:

- Raw extract was prepared by mixing the fish muscles in 0.1MPBS (pH 7.2)and followed by the overnight blending.
- Then centrifugation (10000 rpm, 20 min) was done and collected the supernatant.
- Boiled extract was prepared by boiling raw muscles with 0.1MPBS (pH 7.2) for 10 min at 90°C.
- Then centrifugation (10000 rpm, 20 min) was done and collected the supernatant.
- Later, the fish muscles were fried for 5 mins with mustard oil and oil was eliminated by stuffing on the filter paper. .
- Oil removed fish muscles were mixed with PBS (0.1 M) and centrifuged at 10,000 rpm for 20 mins.
- Every one of the extracts were saved at 20°C.
- Then the concentration of protein in the extracts were determined by Lowry Method.

3.2 Protein Estimation by Lowry Method:

Table 1: The standard protocol for protein estimation by Lowry Method

	BSA Conc (ml)	Distilled water (ml)	Reagent A (ml)	Incubation (10 mins at 50°C)	Reagent B (ml)	Incubation (10 mins at Rt)	Reagent C (ml)	Incubation (10 mins at 50°C)	Absorbance (nm)
	0	1	0.9		0.1		3		0.137
	0.2	0.8	0.9		0.1		3		0.167
	0.4	0.6	0.9		0.1		3		0.170
	0.6	0.4	0.9		0.1		3		0.202
	0.8	0.2	0.9		0.1		3		0.210
	1	0	0.9		0.1		3		0.265
Raw	1	0	0.9		0.1		3		0.278
Boiled	1	0	0.9		0.1		3		0.546
Fried	1	0	0.9		0.1		3		0.878

3.3 Identification by Skin Prick Test:

- With raw, boiled and fried extracts, this test was performed.
- Each extract were mixed in the solution of PBS-Glycerol (1:20 w/v) for example 1ml of PBS:20ml 0f glycerol.
- 10 ul of each concentrate was put on the volar part of the lower arm.
- The skin response was estimated following 20 minutes.

3.4 Quantification by SDS-PAGE

- 10% separation gel was prepared by addition of H_2O (4.1 ml) to acrylamide (3.3 ml, 30%), Tris-HCl (2.5 ml, pH 8.8), 10% SDS (100 µl), TEMED (10 µl) and APS (32µl, 10%).
- Then the gel was poured, leaving 2 cm underneath the look over for stacking gel.
- The highest point of the gel was layered with distilled water.
- Distilled water was eliminated.
- Then 4% stacking gel was prepared by addition of H_2O (6.1 ml), acrylamide (1.3 ml, 30%), Tris-HCl (2.5 ml, pH 6.8), 100 ul SDS (10 %), TEMED (10 µl) and APS (100 µl, 10%).
- Stacking gel was poured on top of the separation gel.
- Combs were added to make wells.
- Gel was braced into device, and loaded up with both support chambers with gel running cushion.
- Samples (raw, boiled and fried extracts) were mixed with sample buffer with SDS (10%), β-mercaptoethanol (5%), glycerol (40%), and bromophenol blue (0.1%) in Triscushion (pH 6.8) and processed for 5 mins at 90°C.
- Tests were stacked.
- Protein bands were envisioned.

CHAPTER 4

Results and Discussion

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4.1 Preparation of Fish Extracts

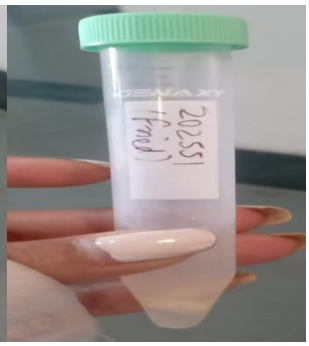

Fig 1: Raw extract **Fig 2: Boiled extract** **Fig 3: Fried extract**

4.2 Protein estimation by lowry method

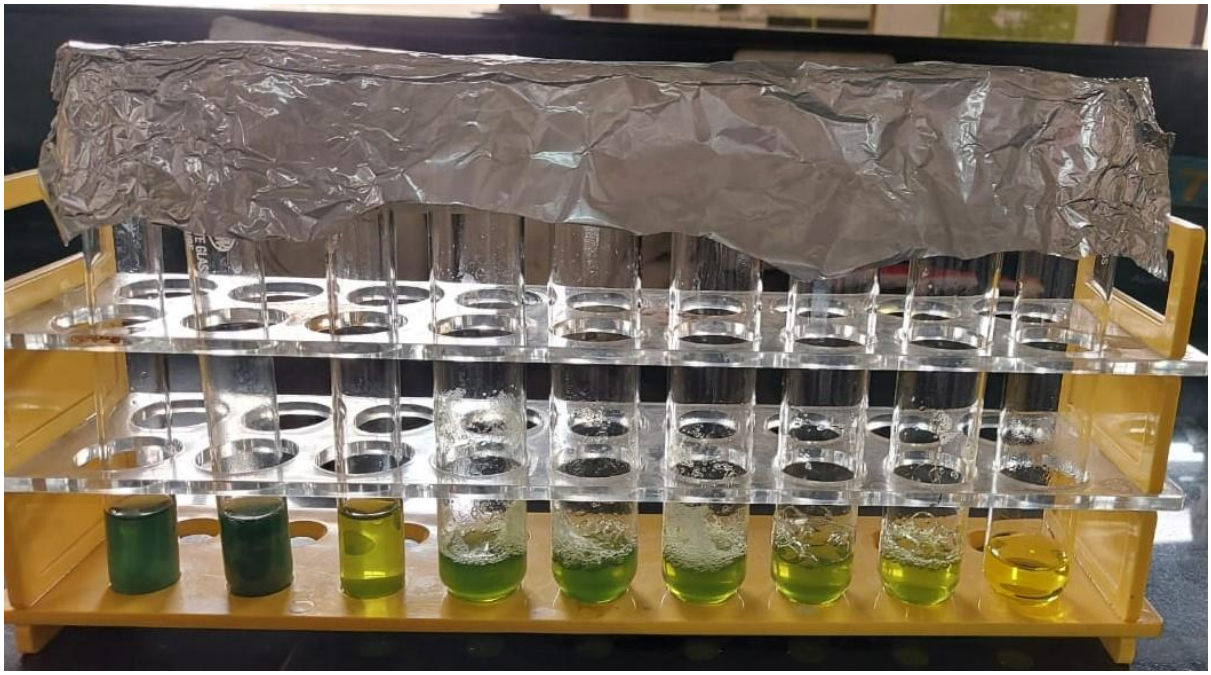

Fig 4: Experimental setup for protein estimation by Lowry Method

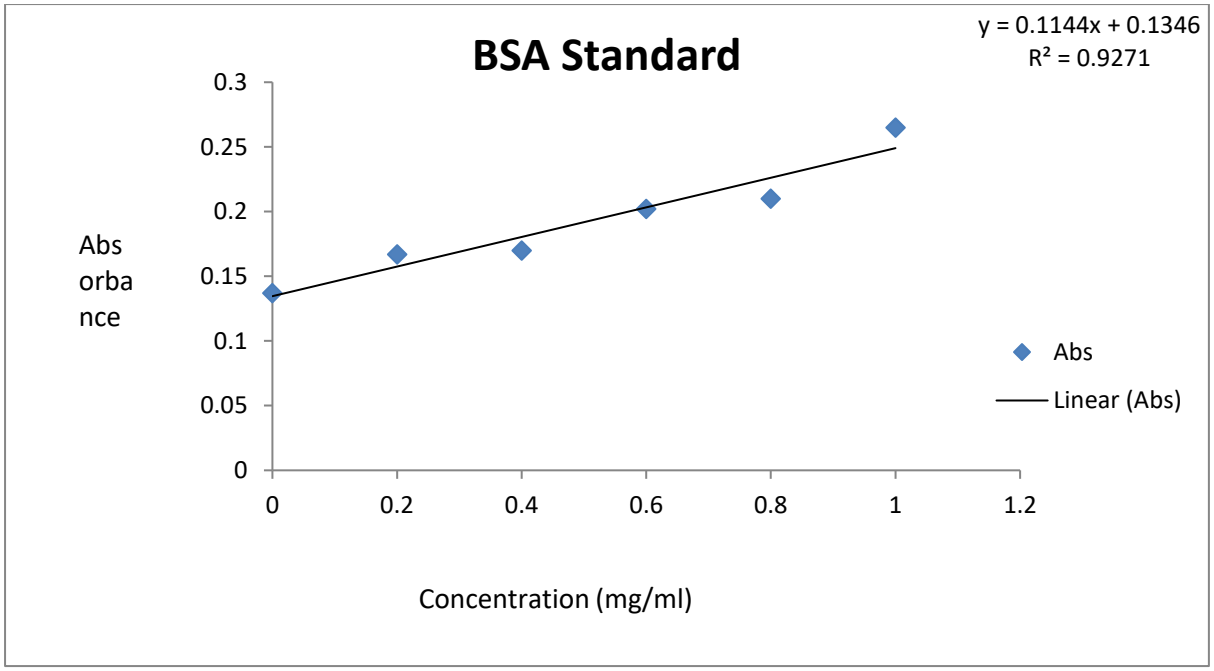

Fig 5: The standard curve for BSA for the estimation of protein in the fish extracts

Calculations:

Y= Absorbance of extract

X= Concentration of extract

Raw extract:

Y=mx+c

0.278=0.1144x+0.1346

X= 1.253

Boiled extract:

Y=mx+c

0.546=0.1144x+0.1346

X= 3.596

Fried extract:

Y=mx+c

0.878=0.1144x+0.1346

X=6.498

4.3 Identification by Skin Prick Test:

The SPT was done on 3 students. Out of 3 students, only 1 student gave positive result with SPT. The patient was subjected to SPT with fried extract. The positive SPT result showed skin rash and redness of the skin.

4.4 Quantification by SDS-PAGE:

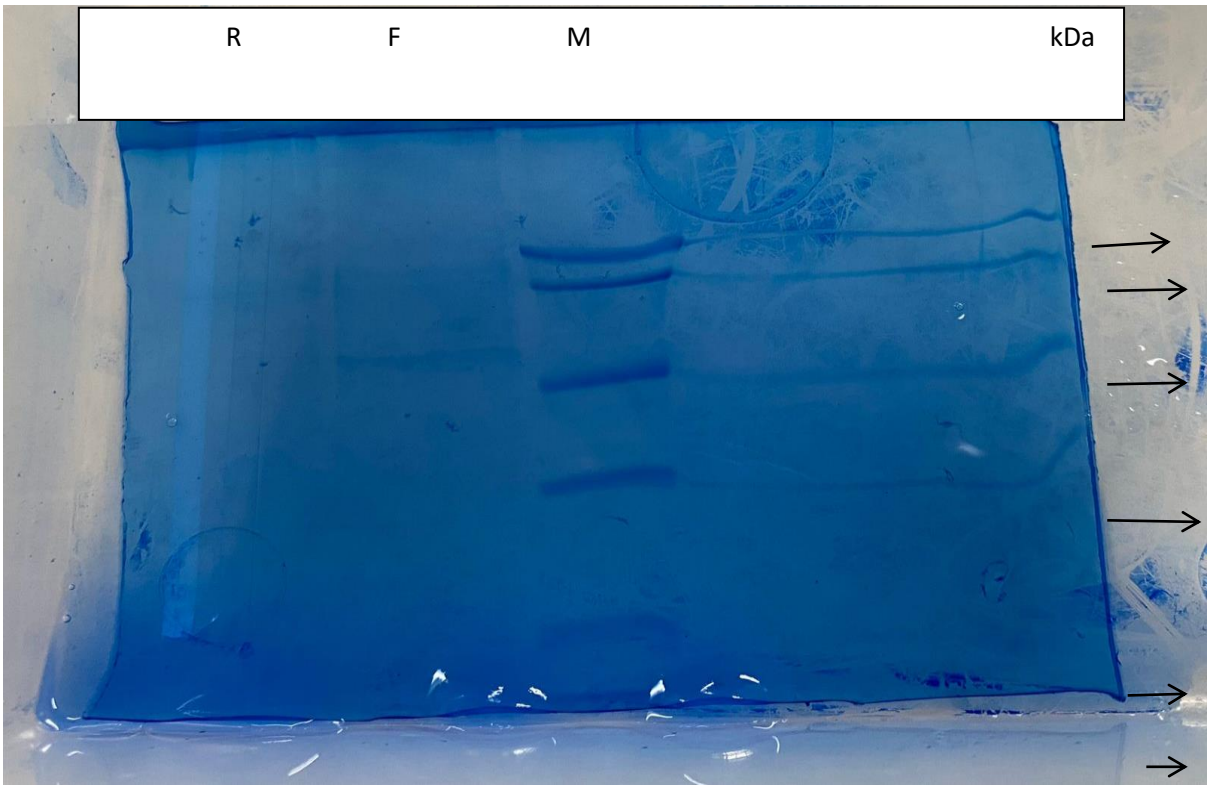

Fig 6: The results for SDS-PAGE gel

The marker was from 20 to 100 kDa. The raw extract of pomfret showed allergenic band at 75kDa. The fried extract of pomfret showed allergeic bands at 100, 75 and 50kDa. The power of food responsive qualities is extending by and large and in this manner tends to a creating general prosperity concern. Governing bodies guarantee ominously powerless purchasers by coordinating the naming of food things containing anticipated allergens. As of now in excess of 600 particular food allergens are known, which shows the collection of existing allergens and the troublesomely in allergen examination and coming about food naming. Until this particular moment, the most typical quantitative strategy for allergen assessment is the SDS-PAGE. The created SDS-PAGE technique was assessed to profoundly be dependable by both repeatability and recuperation tests and effectively applied to quantification.

CHAPTER 5
Conclusions

This research is the primary endeavor to analyze the degrees of allergenicity in crude and thermally handled concentrates of pomfret. Fish is perhaps the most well-known food causing sensitivity, particularly in beach front nations. The major fish allergen in pomfret is enolases with 50kDA and higher molecular weight. Because there is no uniform rule regarding the effects of heating treatment on allergenicity, it is concerned with how a food allergen's ImmunoglobulinE-constricting capacities are altered as a result of cooking. Any manipulation can both create and remove allergic epitopes, such as foaming, searing, frying, salting, drying, or freezing.As a result, determining if and how heat treatment alters a food's allergenicity is a difficult task. Cooking can trigger the Maillard reaction, which raises the allergenicity of some food groups, such as nuts. Warming decreases the allergenicity of cow's milk, yet doesn't absolutely get rid of it. Warming can sometimes completely remove the allergenicity of chitinase-containing foods. Surprisingly, cryptic lipid transfer proteins have a higher allergenic potential and are more resistant to pepsin treatment and warm handling. Broiled planning created significantly distinct SDS-PAGE patterns than crude concentrations, according to our findings. Various groups that were present in the broiled concentrate of Bramidae (pomfret) vanished in the crude arrangement, whereas the seared extract revealed the presence of some new high subatomic weight groups. Overall, our data demonstrated that depending on the individual's ImmunoglobulinE reactivity to the protein in question, gurgling or searing can either diminish or increase allergenicity. The allergenicity of commonly consumed Indian fishes is also included in this study implying thatsome fish allergens aren't as stable as they appear to be when heated.

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