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Protease inhibitors: Its effect on striped catfish (*Pangasianodon hypophthalmus*) surimi shelf life during frozen storage

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Abstract
Surimi, a processed fish product, is widely utilised for making seafood delicacies such as imitation crab and fish balls. Endogenous enzymes found naturally in fish can contribute to surimi breakdown if they are not properly controlled during processing and storage. Enzymes like proteases and lipases can alter the texture, flavour, and overall quality of the surimi product over time. Protease inhibitors produced by legumes can be utilized to reduce surimi degradation due to endogenous proteases. Soybean extract and many legume seeds contain compounds called phytochemicals and peptides that may have antibacterial properties. The effect of a protease inhibitor derived from soyabean on fish muscle homogenate found that the sample has 72.19±0.68% protease activity at room temperature and a protein content of 8.83±0.60 g/ml. However, with the addition soyabean extract the gel strength of surimi was found to be increased significantly and the other functional characteristics of striped catfish surimi improved during the frozen storage. But after 4th month of storage the decreasing trend was observed in all the parameters. Throughout the storage period, high gel strength values were observed in surimi treated with soyabean extract. SDS-PAGE image with the increase in the storage period, MHC band intensity reduced, while there was no change in the actin bands. The surimi added with protease inhibitor (Soyabean extract) also maintained good physio-chemical properties than untreated surimi during period of 6th month of which was due to the protease inhibition and anti-microbial properties of soyabean extract.

Keywords: Protease inhibitors, soyabean, surimi

Introduction
The term "Surimi" refers to the wet concentrate of myofibrillar proteins that is extracted from fish muscle (Okada, 1992) [21]. The fish meat is first deboned, skinned and the extracted mince washed repeatedly with cold water (5 °C), then mixed it with cryoprotectants like salt, starch, and sometimes egg whites or other seafood extracts Vilhelmsson (1997) [20]. Rich in myofibrillar protein, surimi has a light color, a neutral odor, and low fat. It is an especially functional food because of the distinct gelling property of the myofibrillar proteins. Therefore, surimi is an excellent ingredient to use for the preparation of analog and imitation products Lanier (2000) [14].

In surimi preparation washing process have a great importance, due to washing fat, unwanted materials such as pigments, blood and odour was removed and due to this there is increase in the concentration of myofibrillar proteins, which helps to enhance the gelling property of surimi Lanier & Lee (1992) [15].

According to Sankar & Ramachandran (2002) [25] freshwater fishes, especially major carps, are good to use to prepare mince and surimi. When comparing surimi from marine species to that from freshwater fish species, Luo *et al.* (2001) [16] found that the gel characteristics of the freshwater fish surimi are lower. Nousad *et al.* (1999) [19] reported that surimi from tropical freshwater fish species, including snakehead, wild mullet, and Nile tilapia, showed good gel setting ability. In many parts of India, farmers are now choosing to farm catfish instead of carp, which is increasing the overall production of fish Tanuja. S. *et al.* (2014) [27].

Formation of ice crystals reduces the quality of surimi; these ice crystals can form in the critical temperature range of -1^0 to -5 °C. Matsumoto and Noguchi (1992) [17] explain that this can be avoided by freezing the product rapidly or by minimizing the duration of time it spends in this temperature range. The quality of surimi is affected by various factors such as the rate of freezing, temperature during storage, stability of storage conditions, and thawing conditions. The texture of seafood products made from surimi is impacted by the fish's muscle integrity during storage. One of the primary factors of fish muscle disintegration and an adverse texture impact on surimi-based products is endogenous proteinase. In commercial surimi production methods, it is difficult to eliminate cathepsin B, L, and L-like protease because it reduces actomyosin and directly affects surimi gelation during the synthesis of seafood analogues. In order to prevent the negative effects caused by proteolytic activity, protease inhibitors have been used extensively.

The most commonly used inhibitors are potato powder, egg white, whey protein concentrate, and beef plasma protein (BPP). Plant-based foods like fruits, seeds, and tubers have been found to contain several types of protease inhibitors; these foods also tend to contain significant amounts of proteinase inhibitors.

Plant-derived protease inhibitors that content proteins and peptides, and these peptides are have the ability to stop proteolytic enzymes from catalysing their own reactions. By combining with the target protease to form a stable structure, these inhibitors prevent, alter, or stop the enzyme's active site from being accessible. Extracts of soyabean and other leguminous seeds have been found to contain serine protease inhibitors, which have attracted significant attention (Bhattacharyya *et al.*, 2006; Sriket *et al.*, 2011) [7, 26]. The current research objective was to find out whether protease inhibitors extracted from legume sources may increase surimi's shelf life while stored in its frozen condition.

Material and Methods

Extraction of proteinase inhibitors from legume seeds

Legume seeds were purchase from KVK, Shirgoan and these seeds were dried and grounded by using grinder. Hexane was used to defat the seed flour (5 ml/g) for 10 minutes. The mixture was filtered using Whatman No. 1 filter paper to remove any remaining oil, and the sediment was then washed three times with hexane. The defatted flour was allowed to air dry at room temperature until it was completely dry and odourless. To extract legume seed proteinase inhibitors, distilled water was utilised as the extraction medium. After adding the defatted seed flour (3 ml/g) to the medium, it was agitated for an hour at room temperature at 150 rpm. The extract was recovered by centrifuging at 5,000×g for 30 minutes. The clear solution was kept at 4 °C until use.

Protein determination

According to the method explained by Gornall *et al.* (1949) [10], using the biuret method and BSA (bovin serum albumin) as a standard, Protein determination of samples was estimated.

Proteinase inhibitor assay

The inhibition of muscle protein proteinase was performed according to the method of Benjakul *et al.* (1996) [2]. Diluted proteinase solution (100 µl) was incubated with 100 µl of legume seed proteinase inhibitor extract for 20 minutes at ambient temperature. The residual proteinase activity was measured using casein as substrate in 50 mM Tris-HCl buffer pH 7.5 with 40 mM $CaCl_2$. The assay of muscle protein was conducted as the percentage of proteinase activity inhibited:

$$\text{Inhibitory activity (\%)} = \frac{\text{tyrosine released (without LSPI)} - \text{tyrosine released (with LSPI)}}{\text{tyrosine released (without LSPI)}} \times 100$$

Protein solubility

The protein solubility of surimi samples was measured accordingly to the method described by Rawdkuen *et al.* (2009) [24]. Weigh 2 g of sample and homogenized (‘Remi motor’, homogeniser of RPM 8000) with 18 ml of 0.5 M borate buffer solution, pH 11.0, for 60 sec and stirred for 30 min at 4 °C. The homogenates were centrifuged (Hettich Zentrifugen, D-78532, Germany) at 8000 rpm for 5 minutes at 4 °C, and the protein concentrated of supernatant was measured by the Biuret method. Protein solubility (%) was defined as the fraction of protein remaining soluble after centrifugation and calculated as followed:

$$\text{Protein solubility (\%)} = \frac{\text{Protein concentration in Supernatant}}{\text{Protein Concentration in homogenate}} \times 100$$

Preparation of striped catfish surimi by conventional method: Freshly caught striped catfish (*Pangasianodon hypophthalmus*) with the size of 1-1.5 kg were procured from local aquaculture farm located, Chiplun, Maharashtra and transported to laboratory in polystyrene foam boxes filled with ice. Fresh fish was subjected to deheading and degutting manually and mince was separated using deboning machine (BADDER 600) with a 3 mm perforated drum. Mince was packed in a plastic bag and kept in the polystyrene foam boxes filled with ice (4 °C) for further study. Conventional surimi was prepared according to the method given by Chaijan *et al.* (2010) [8]. The fish mince was washed with cold water (4 °C), using water to mince ratio of 3:1 (v/w), the mixture was stirred gently for 10 minutes and the washed minced was filtered with a layer of cheese cloth, and dewater it by squeezing. Washing was preformed three times. Third washing step was carried out with 0.5% Nacl solution, the ratio of mince to Nacl solution was 1:3 (w/w). Cryoprotectants such as 0.2% sodium tripolyphosphate and 4% sucrose were added to washed mince. The surimi was frozen in plastic bag at -40 °C and stored at -20 °C.

Preparation of surimi with protease inhibitor

Protease extract was added to conventional striped catfish surimi in 1:1 proportion of aqueous extract at a concentration of 2% (v/w) of the surimi.

Surimi gel preparation

The heat-induced surimi gel was prepared according to method of given by Balange and Benjakul (2009) [1].

Partially thawed surimi was smashed with 2.5% salt for 3 min at 4 °C to obtain a homogeneous solution. It was then stuffed into poly-vinylidene casing with a diameter of 2.5 cm and both ends of the casing were sealed tightly.
Sols were incubated at 40 °C for 30 minutes, followed by heating at 90 °C for 20 minutes in a water-bath and that sample was referred to as kamaboko gel Chaijan *et al.* (2010) [8]. All gels were cooled in iced water for 20 minutes and stored overnight at 4 °C prior to analysis.

Determination of Gel strength
Gels were tested at room temperature. Prepared surimi gels were cut into cylindrical pieces of 2.5 cm length. The breaking force (gel strength) and deformation (elasticity/ deformability) were measured for each sample by keeping the pieces of each sample into the texture analyzer equipped with a probe a having diameter of 5 mm and speed of 60 mm/min spherical plunger. The probe was pressed into the cut surface of gel perpendicularly at a constant speed, until punctured. The force in gram (g) required to puncture into the gel (breaking force) and the distance (mm) at which the probe punctured into the gel (deformation), were recorded. Gel strength for each surimi gel was calculated from respective breaking force and deformation.

Whiteness
Whiteness of surimi samples was determined by using whiteness meter. The measurement of L* (lightness), a* (redness/greenness) and b*(yellowness/blueness) was performed in triplicate. Whiteness was calculated as per the following formula given by Park *et al.* (2006) [22].

$$\text{Whiteness} = 100 - [(100\text{-}L^{*})^2 + a^{*2} + b^{*2}]^{½}$$

Expressible moisture content
Expressible moisture content was determined according to the method of Rawdkuen, S. *et al.* (2009) [24]. Surimi gel samples were cut to a thickness of 0.5 cm was weighed (X) and placed between two and three sheets of Whatman paper no. 1 filter paper at the top and bottom of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The sample was then removed from the papers and weighed again (Y). The expressible moisture content was calculated using following formula:

$$\text{Expressible moisture content (\%)} = \frac{(X - Y)}{X} \times 100$$

SDS-PAGE gel electrophoresis
The band pattern of protein obtained from different methods was determined by using electrophoresis according to method of Laemmli (1970) [13]. Three grams of surimi sample was homogenized with 27 ml of 5% (w/v) SDS for 1 min at the speed for 11,000 rpm. The homogenate was incubated at 85 °C for 1 h to dissolved total proteins, followed by centrifugation at 3,500 × *g* for 20 min at room temperature, to remove undissolved debris. The 20 µl supernatant were mixed with 5 µl sample loading buffer and boiled the tube containing protein sample at 100 °C for 3 min in a boiling water bath. The sample (20 µl protein) was loaded into polyacrylamide gel subjected to electrophoresis at a constant current of 110 volts. After separation the protein were stained with 0.02% (w/v) Coomassie Brillant Blue R-250 and destained with solution containing 50% distilled water (v/v) and 40% (v/v) methanol and 10% (v/v) acetic acid.

Statistical analysis
All experimental data analysis was done in SPSS 16.1 software, using one-way ANOVA and Duncan's multiple range tests for each data group. Significant differences in means were also determined, and comparisons were made at the 5% confidence level. A p value of less than 0.05 was considered statistically significant, and the final results were reported as mean±standard deviations.

Result and Discussion
Assay of protease activity and its inhibition by legume seeds extracts
The use of protease inhibitor to control proteolysis and the resultant softening in the surimi during frozen storage was evaluated by treating the surimi with legume seed extract. Various legumes seeds showed different inhibitory activity against the muscle protein of striped catfish, and the protein concentration of legume seeds were estimated. The result shows the highest inhibitory activity was observed in soyabeans legume seed extract 72.19±0.68% with protein concentration was 8.83±0.60g/ml. whereas, the highest protein concentration was observed in white cowpea 10.49±0.58 g/ml with lowest inhibitory activity ($p \leq 0.05$). Benjakul *et al.* (2000) [4] studied the inhibitory effect of legume seed extract on fish proteases were extract from black cowpea (95.63±1.56%) and soyabean (84.11±0.59%) showed high inhibitory activity against the muscle protein. Garcia-Carreno *et al.* (1996) [9] found that seed extract inhibited papain and trypsin activity and reduced proteolysis of pacific whiting and arrowtooth flounder. Benjakul *et al.* (2000) [4] showed that the addition of a partially purified proteinase inhibitor from cowpea, pegion pea, Bambara groundnut at the specific activity of 13.48, 15.23, 6.77, 7.70 Kunits /mg of proteins respectively. All inhibitor activity shows against proteases from threadfin bream which are responsible for Modori inducement, reduced autolytic degradation and improved textural properties of threadfin bream muscle proteins. However, no information is available on the nature of inhibitory compounds and their characteristics from these sources.

Table 1: Inhibitory activity and protein concentration of various legume seed extracts

Legume seed	Inhibitory activity (%)	Protein concentration (g/ml)
Soyabean	72.19±0.68[1]	8.83±0.60[3]
White cowpea	20.88±0.72[2]	10.49±0.58[1]
Dolichos bean	52.28±1.10[4]	7.06±0.31[2]
Mung bean	47.13±1.01[3]	6.48±0.43[2]

From, the present study it was found that when soyabean extract was used as protease inhibitor it shows prevention of autolytic activity but when used with surimi it also shows significant increase in gel strength of surimi. The protein

concentration of the extract was observed to be 8.83±0.60 ml/g of soyabean seed. Protease activity of soyabean extract was carried out; the extract was added to conventional surimi in 1:1 proportion of aqueous soyabean extract at a concentration of 2% (v/w) of the surimi. Further its effect on the functional properties of surimi such as solubility, gel strength, whiteness, and expressible moisture content etc., were evaluated.

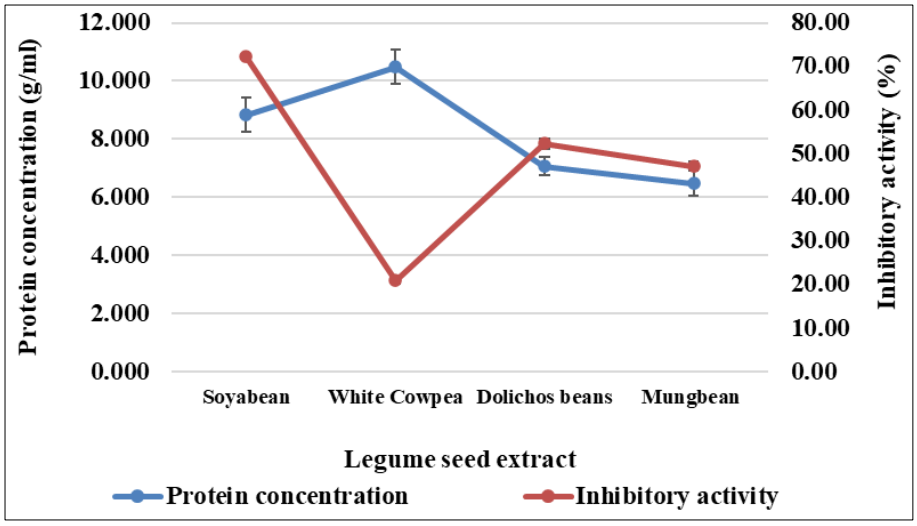

Fig 1: Inhibitory activity and protein concentration of various legume seed extracts

Solubility

Protein solubility often affects functional properties and the most affected are thickening, foaming, emulsifying and gelling of proteins Zayas, J.F. (1997) [28]. Changes in solubility values of surimi of control and with treated surimi during six months of frozen storage. During frozen storage the solubility of both the samples decreased, but surimi treated with soyabean extract gave higher solubility as compared to control surimi sample. The extract treated surimi sample had higher value of solubility 82.34±3.52% on 30th day of frozen storage than the initial value of treated surimi sample. The solubility then decreased to 79.79±1.10%, 77.93±0.81%, 75.77±2.94% and 68.18±1.92% on 60th, 90th and 180th day of frozen storage respectively. The solubility values of control as well as surimi treated with soyabean extract decreased with increase in frozen storage time and there was a significant difference between the samples (p<0.05). The high solubility values of all the surimi samples confirm that there was very little freeze-induced protein aggregation even in control sample Zayas, J.F. (1997) [28]. The high solubility in the surimi samples can be due to the high-water holding capacity. The amino acid residue affects its solubility characteristics. The addition of legume seed extract to surimi increases its protein solubility for a number of factors; as legume seed extracts contain a variety of components, including specific proteins, peptides, and polysaccharides, which work as solubilizing compounds and can disrupt protein-protein interactions in the surimi matrix, resulting in protein molecule dispersion and increased solubility. Several proteins in legume seed extracts contain hydrophilic (water-attracting) region that allow them to interact with water molecules in surimi. This interaction can improve protein dispersion in the aqueous phase, resulting in higher protein solubility. Legume seed extracts have the ability to change the pH and ionic strength of surimi. Such changes may have an effect on the electrostatic and hydrophobic interactions between proteins, influencing their solubility.

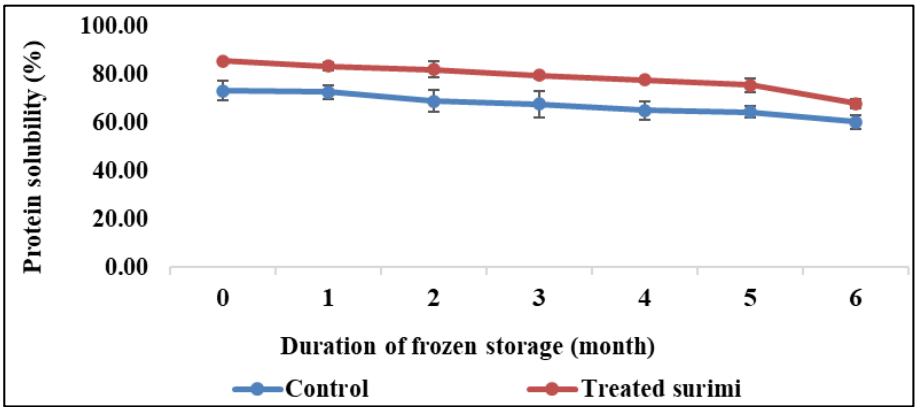

Fig 2: Protein solubility (%) of control and treated surimi with protease inhibitor during frozen storage (Error bar indicates the SD)

Gel strength

The gel strength of surimi treated with soyabean extract was compared with control surimi, comparatively lower values of gel strength observed in the control sample must have been due to the effect of proteases that are responsible for modori inducement. The results show that the surimi treated with soyabean extract had higher gel strength as compared to that of control sample. The gel strength of extract treated

surimi gel was higher than untreated sample. The initial gel strength of control sample was 400.58 g/cm and the surimi treated with soyabean extract was 420.95 g/cm.

The increased gel strength values of the treated sample could be attributed to the soyabean extract's inhibition of modori causing protease. Surimi gels preincubated at 40 °C and 60 °C prior to heating at 90 °C to observe the inhibited autolytic breakdown. Preincubation of surimi at 35-40 °C in the presence of salt is performed for the high temperature setting or "suwari". The cross-linking of myosin heavy chain can be accelerated by endogenous transglutaminase, resulting in the enhanced gel properties. Benjakul *et al.* (2000) [4] found that incorporation of the partially purified proteinase inhibitors from legume seeds markedly decreased the extent of autolytic degradation in surimi gels prepared by incubating at 40 °C or 60 °C prior to heating at 90 °C. Kudre and Benjakul (2013) [12] explained the application of protein isolates the breaking and deformation force of both kamaboko and modori gels increased. When the MBPI or BBPI, or BGPI at a level of 1.5% was mixed, breaking force of kamaboko and modori gels increased by 22.4-76.8 to 90.0-135.3%, while deformation increased by 11.6-31.3 to 26.4-61.39%. As the concentration of MBPI, BBPI and BGPI increased, myosin heavy chain (MHC) of modori gel was retained more. The effect of BGPI exhibited the superior gel enhancement effect than BBPI and MBPI. Therefore, BGPI at an appropriate level could be an alternative food grade protein additive to improve gel properties of surimi. During the frozen storage the gel strength of both the samples deceased but, surimi treated with soyabean extract gave higher gel strength compared to control surimi. However, the compounds present in the extract and their role in strengthening of gel strength of surimi is not clear.

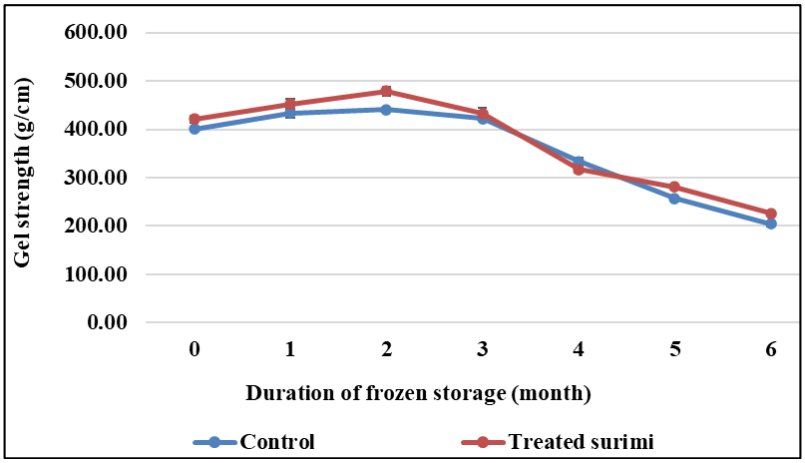

Fig 3: Gel strength (g/cm) of control and treated surimi with protease inhibitor during frozen storage (Error bar indicates the SD)

Whiteness

The whiteness of surimi gels was depending upon the type of legume seed protease inhibitor added; here the whiteness of treated surimi gel was decreased as compared to control surimi gel. Park, (2013) [22] reported that a decrease of L* value with increase in b* value of pacific whiting surimi gel added with 1% beef plasma protein (BPP). There was no significant difference in whiteness values. The colour quality of surimi gels were significantly affected by the types and amount of additives used (Benjakul *et al.*, 2000; Rawdkuen *et al.*, 2008) [23, 4]. During the frozen storage all the surimi samples shows decrease in whiteness values. However, the decrease in the whiteness was not affected upto 4th month; thereafter the values show the decreasing trend in all samples. The resulting decrease in whiteness values was most likely caused by the abduction of pigment proteins, specifically oxidized pigments, to muscle proteins. Nopianti *et al.* (2011) [18] and same reasons also given by Jaimie *et al.* (2005) [11] that to presence of contaminated indigenous brown or black coloured pigments in legume seed protein isolate from seed coat during extraction of proteins responsible for decrease in whiteness of surimi.

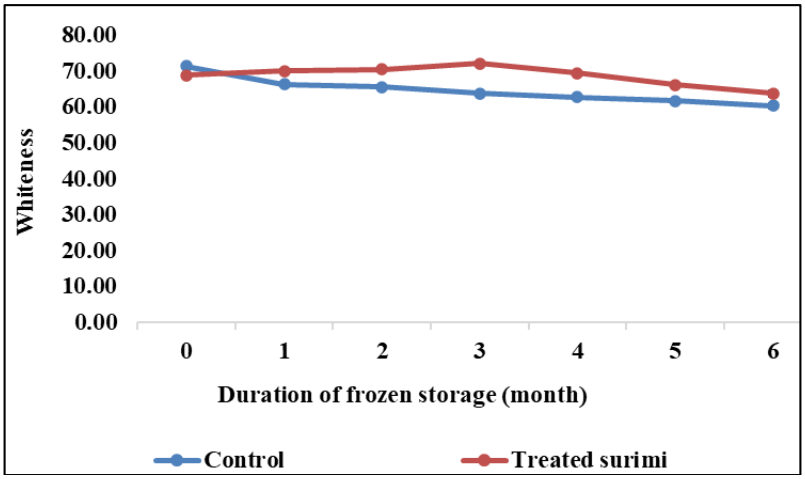

Fig 4: Whiteness values of control and treated surimi with protease inhibitor during frozen storage (Error bar indicates the SD)

Expressible moisture content

The expressible moisture content of treated surimi gel was increased with the addition of soyabean extract. However, during frozen storage there was no significant differences value of treated and control surimi samples. The increase in expressible moisture during frozen storage may be due to extent of protein denaturation induced by the frozen storage that has low affinity for water Benjakul *et al.* (2004) [5].

The continuous denaturation of protein in other species during frozen storage also resulted in a lower water holding capacity, resulting in a decline in expressible moisture content. Furthermore, freezing causes the development of ice crystals, which can cause tissue damage and the release of different organelles. As a result, water could be released from muscle more easily, particularly when the frozen storage time increased Benjakul *et al.* (2003) [3].

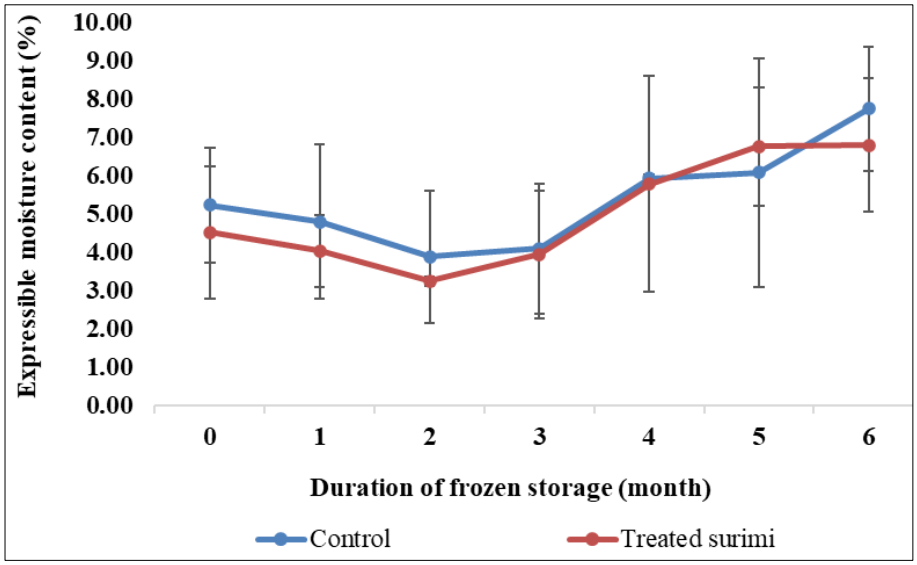

Fig 5: Expressible moisture content of control and treated surimi with protease inhibitor during frozen storage (Error bar indicates the SD)

SDS-PAGE of surimi proteins

The SDS-PAGE image for control and treated surimi samples for frozen storage after every two months are given in image fig 2. Myosin light chains with a molecular weight of 200-180 kDa were seen, as well as an actin protein band that was prominently observed and resolved with improved band intensity in all surimi samples examined for varying months of frozen storage. Additionally, soyabean extract added surimi samples exhibited a protein band near 48kDa identified as band intensity was not as prominent as actin. With increase storage period few a greater number of bands was seen in samples. Decrease in myosin heavy chain bands in untreated has been observed when compared with treated samples which could be due to formation of cross-linking by SS bonds that stabilized the myosin heavy chain during setting Benjakul and Visessanguan (2003) [3]. In the present study, as seen from with the increase in the storage period, MHC band intensity reduced, while there was no change in the actin bands.

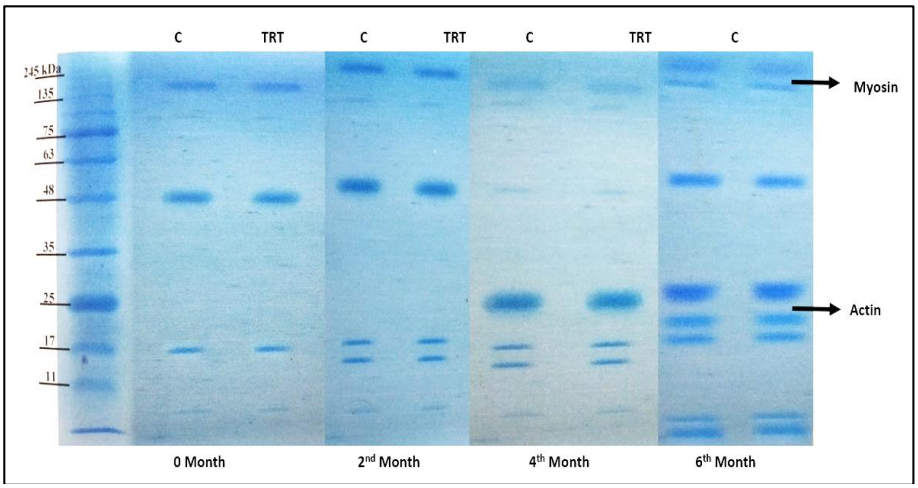

Fig 6: SDS-PAGE protein pattern of control and treated surimi with protease inhibitor (Soyabean seed extract) and the effect of proteolytic activity for 6 months during frozen storage

Conclusion

Legume seed extracts of soybean, contain various proteins and functional compounds. The potential of this extract is to improve the functional properties of surimi, and other fish-based product. The addition of legume seed extracts to enhance the texture, gel-forming ability, and overall quality of surimi. It is essential to note that the particular mechanisms and effects of legume seed extracts on surimi and its functional properties can vary depending on the type

of legume utilized, the processing conditions, and the interactions with other surimi ingredients.

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