J Food Sci Technol (January 2020) 57(1):1–11
https://doi.org/10.1007/s13197-019-04044-6

REVIEW ARTICLE

Inhibition of post-mortem fish muscle softening and degradation using legume seed proteinase inhibitors

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Revised: 29 July 2019 / Accepted: 19 August 2019 / Published online: 24 August 2019

Abstract Inhibitors that control muscle softening are important for regulating the activities of specific proteinases in meat. Proteolytic activity of endogenous proteinases in postmortem fish leads to the deterioration of myofibres. Calpain proteolytic enzyme system in skeletal muscles is mainly responsible for the post-mortem proteolysis. Soluble sarcoplasmic serine proteinase and the insoluble myofibrillar serine proteinase fractions contribute to the modori effects in surimi gels while myosin heavy chains contribute to gel strength. Proteolytic degenerative processes negatively affect the entire quality spectrum of the fish as food. Legume seeds are a good source of proteinase inhibitors with the potential to emerge as a promising tool in fish meat quality management. Many workers have studied the potent inhibitory effect of the seed flour from various legume crops on the flesh, surimi gels and visceral proteinases of fishes. The present review provides collective information about proteolysis in fish and its control by using legume seed flour as a natural source of proteinase inhibitors. Use of legume seed flour can reduce the dependence of the meat processing industry on the non-renewable synthetic chemical agents. Moreover, the use of natural products from sustainable resources also leads to the improved economics of meat production.

Keywords Proteolytic inhibitors · Softening · Legume seeds · Endogenous proteases · SERPINS

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Introduction

Meat is recognized as nutritious food rich in amino acids, vitamins (B_{12} and other B complex components) and minerals (iron, selenium, zinc, and phosphorus) essential for human diet (Pereira and Vicente 2013). It is the richest source of high-quality proteins and is a preferred dietary source of animal proteins consumed worldwide (Lafarga and Hayes 2014). The consumer demands for the meat and seafoods were increased substantially in recent times due to the rise in the human population (Singh and Benjakul 2018). By 2050 an expected 30% rise in the population resulting in 9×10^9 humans on the earth will create a demand for approximately 365×10^6 tonnes of dietary proteins that otherwise may lead to global malnutrition and starvation (Béné et al. 2015). The other most important factor responsible for creating excessive huge demands for seafoods in the near future will be climate change by way of global warming. This may decrease the crop production potential of soil and pose a significant threat to food production as well as food security. The growing demand for seafoods may be overcome by increased fish production as the oceans, lakes, and rivers collectively will have to feed the ever-growing human population (Béné et al. 2015).

'Softening' is an important term in meat and seafoods that refer to the post-mortem degradation of muscle food due to the hydrolytic activity of certain endogenous proteases (Sriket et al. 2011a). It involves the myofibrillar proteolysis leading to the muscular disorganization. In muscle foods, the myofibrillar proteins are largely responsible for the maintenance of required textural and the sensorial characteristics. Fish muscle softening during iced storage has been reported in the red sea bream (*Pagrus major*) (Wu et al. 2010), common carp (*Cyprinus carpio*) (Wu et al. 2008), Mexican flounder (*Cyclopsetta*

chittendeni) (Ramírez et al. 2002), etc. Myosin heavy chain (MHC) component in myofibres is observed to be primarily affected (Hu et al. 2010). The process of minimizing the protease-mediated muscle protein degradation is necessary to maintain fish meat quality for prolonged durations (Sriket 2014). The conventional preservation techniques (icing or refrigeration) during storage employ certain additives from animal sources (whey protein, egg white, chicken and Porcine blood plasma) that prevent fish softening as well as surimi gel weakening to certain extents (Singh and Benjakul 2018). Studies have claimed that certain plant products (e.g. legume seed flour) are capable of controlling the proteolytic degradation in fish and fish products in more acceptable and preferred ways (Singh and Benjakul 2018).

Legume seeds are an excellent source of dietary components owing to their health-promoting effects and nutritional properties (Singh et al. 2017a, b, c). Legume seeds have a significant amount of protease inhibitors (Martín-Cabrejas et al. 2009). Studies have tested the impact of legume seeds on the proteolytic degradation of fish and proved the potential of legume seeds to be utilized as an effective strategy to alleviate the softening related problems faced by the fish meat industry (Singh and Benjakul 2018; Sriket et al. 2011b; Benjakul et al. 1999; García-Carreño et al. 1996). The present scenario of the food processing industry involves the research and development of newer beneficial functional aspects of the legume seeds (Singh et al. 2017a, b, c). In light of the above, the current review is proposed on the use of legume seeds in meat industry to maintain the quality and shelf life of fish meat products.

Fish as human food

Fish is a vital and integral part of food in a well-balanced human diet (Prato and Biandolino 2015). It represents one of the world's most nutritious and healthy food source rich in vitamins (A, B and D), minerals (calcium, iron, zinc, iodine and selenium) and high-value proteins (Tacon and Metian 2013). Fish meat contains higher protein content (on an edible fresh weight basis) than the terrestrial animal meats (Tacon and Metian 2013). Moreover, fish are characterized by low-fat content and fish protein had an excellent essential amino acid profile for human consumption (Prato and Biandolino 2015). Fish is being promoted as rich food for poor people living along coastal areas (Prato and Biandolino 2015). Being an important source of food proteins, the consumption of fish is continuously increasing (Mazorra-Manzano et al. 2018). The total calculated amount for fish consumption comes out to be 110 million tons with respect to 140 million tons of total fish production (Benhabiles et al. 2012). The consumer demand for fish is expected to increase further in the near future. Fish consumption reduces the risk of cancers, diabetes, cardiovascular and inflammatory diseases (Tacon and Metian 2013; Prato et al. 2019). The health benefits of fish consumption have been associated with the presence of docosahexaenoic acid (DHA) and ω-3 eicosapentaenoic acid (EPA) that occur in large amounts in most fish (Prato et al. 2019). The changes in quality of fish during storage has attracted the attention of consumers and the fish meat industry since it is an integral component of the human diet (Singh and Benjakul 2018; Yu et al. 2019).

Fish softening

Proteins are recognized as important markers of quality in seafoods (Mazorra-Manzano et al. 2018). Fish softening is a common problem in the fish industry. Post mortem fish undergoes softening very rapidly in contrast to mammalian flesh even during cold storage (Chéret et al. 2007) and always remain a major concern in quality aspects (Sentandreu et al. 2002). The steps involved in fish degradation leading to spoilage is shown in Fig. 1. Post-mortem softening is a collective result of a number of several intracellular proteolytic steps (Ouali et al. 2006), which is evidenced to be mediated by various enzymatic reactions (Ghaly et al. 2010). However, in general, the rigor mortis, resolution of rigor, autolysis and bacterial spoilage are the four phases in the fish deterioration process. After death, during storage and transportation, the liberation of digestive enzymes specifically proteases play a key role in rupturing of stomach wall and induction of extensive autolysis (Pushparajan et al. 2013). Endogenous hydrolytic enzymes such as cytosolic calpains, lysosomal cathepsins, connective tissue elastases and collagenases participate in softening of fish muscle by the degradation of proteins (Ahmed et al. 2015). Studies reported calpain-mediated changes in α-actinin in the carp (Tsuchiya and Seki 1991) and action of cathepsins B and L in salmon muscle structural proteins (Yamashita and Konagaya 1991).

The synergy between different cellular proteases is responsible for the postmortem softening indicating the complexity of the process (Delbarre-Ladrat et al. 2004). In spite of a number of studies available in the literature, there are still certain unclear gaps that create a flaw in understanding and revealing the complete mechanism of postmortem proteolysis. However, with the applications of modern proteomic technologies, the researchers are continually revealing the novel insights in this multienzymic process (D'Alessandro and Zolla 2013). Studies involving knockout mice using recombinant DNA technology confirmed the participation of μ-calpain proteases as the

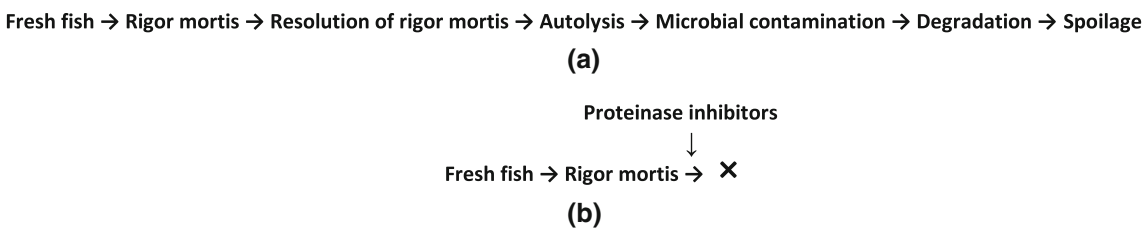

Fig. 1 Steps in fish degradation leading to spoilage. **a** In the absence of proteinase inhibitors, **b** In the presence of proteinase inhibitors

important mediators of postmortem proteolysis. The gene silencing studies involving μ-calpain gene does not achieve complete attenuation of the proteolytic process in the skeletal muscles (Geesink et al. 2006). Thus, studies indicated the involvement of other proteases also in the postmortem proteolysis (Geesink et al. 2006). Despite extensive efforts, the complete mechanisms behind the post mortem fish softening are unclear (Kemp et al. 2010).

Fish myofibrillar degradation

Fish myofibres are composed of 60–80% of total protein contents of which the sarcoplasmic proteins constitute 20–50% and connective tissue proteins (collagen) account for 3–10% (Delbarre-Ladrat et al. 2006). Anatomically connected to the myocommata (connective tissue), the fish muscle cells are arranged parallel to each other along with desmin, spectrin (intermediate filaments), integrin, sarcoglycan (transmembrane glycoproteins), vinculin, dystrophin, vimentin (costamere), actin (thin filament), myosin (thick filament), α-actinin (z-disk), troponins, tropomyosin, nebulin and titin proteins, thereby, constitute the sarcomere (a basic repeating unit of myofibrils). Among all myofibrillar proteins, the myosin is the most abundant, while actin and titin rank second and third, respectively (Delbarre-Ladrat et al. 2006). During storage, the muscle cells along with connective tissue are prone to the action of proteolytic enzymes (Delbarre-Ladrat et al. 2006) and proteolysis of cytoskeletal components leads to myofilament degradation. Degradation of myofibrillar proteins (e.g. α-actinin, connectin in carp and rainbow trout muscles) have been noticed in different fish species. The cleavage of the myosin protein is responsible for the weakening of myofibrillar structures (Hopkins and Thompson 2002).

Degradation of collagen protein (the major extracellular matrix component) includes collagen fibril disorganization, intermolecular collagen cross-links breakdown, increased space between fibers followed by collagen junction cleavage (the connection between myocommata and muscle fibers). Ando et al. (1992) reported collagen fibers disintegration in the pericellular connective tissue leading to changes in the three-dimensional fibrillar network during post-mortem tenderization of rainbow trout muscle. Histological examinations involving the ultrastructural studies of the yellowfin tuna (*Thunnus albacares*), southern bluefin tuna (*Thunnus maccoyii*) muscles and scanning electron microscopic investigations of the muscle fibre connective tissue junctions in the spotted trevalla (*Seriolella punctata*) concluded the proteolysis of muscle structure as the responsible phenomenon in the postmortem muscle-softening (Ishida et al. 2003). It is now an established fact that the myofibrillar protein degradation is a decisive factor in post-mortem proteolysis along with the rate and extent of proteolysis as the essential determinants.

Surimi and modori

Surimi is the washed minced fish gel composed of myosin heavy chain (MHC) and actins as its primary protein components that participates in the formation of Kamaboko gel (heat-induced protein structure) (Kunimoto et al. 2016). Modori is the structure disintegration phenomenon at 50-70 °C that leads to an irreversible deterioration in the texture of surimi characterized by brittle, nonelastic gels showing decreased gel strength. Modori is induced by the protease activity of endogenous thermal stable proteinases (specifically serine-type) on the myosin component of the gel (Yongsawatdigul et al. 2000). Solublizable sarcoplasmic serine proteinase (SSP) and insoluble myofibrillar serine proteinase (MSP) fractions together are evidenced to be the extensive contributors of the modori effects (Cao et al. 1999). Among both fractions, MSP is uniquely referred as modori inducing protease which is solely responsible for the initiation of the degradation process (Ohkubo et al. 2004). The presence of MSP is reported in various fish species viz. Anchovy (*Engraulis japonica*), Carp (*Cyprinus carpio*), Lizardfish (*Saurida wanieso*) and White croaker (*Argyrosmus argentatus*) etc. In myofibrils, the MHC and actinin are the major constituents, while tropomyosin, troponin-T, α-actinin as well as myosin light chains (MLCs) are considered as the minor constituents. MHCs are regarded as the major contributor in the surimi gel strength and they are the most susceptible substrates of MSPs. In MSP mediated proteolysis of carp (*Cyprinus*

carpio) myofibrils, the MHCs hydrolyzed rapidly than tropomyosin/troponin-T, while actin degraded only to some extent and α-actinin degrade minutely (Cao et al. 1999).

Texture

The texture is a general quality trait that is highly associated with fish meat quality and is the major quality factor governing consumer's perception towards the product. State of the muscle fibrils and connective tissue proteins largely governs the meat texture. Both the muscle proteins and the muscle fibers are closely linked to the muscle food hardness. However, the muscle fibrils remain the more important contributor in texture than the connective tissue proteins (Hu et al. 2014). Collagen content is related to the tenderness that also has implications in quality deterioration (Suarez et al. 2005). Two types of collagen proteins i.e. type I and type V present in the intramuscular connective tissue are recognized as the quality (texture and toughness) contributors in muscle food (Alderton et al. 2004). Both type I and type V collagen proteins disintegrate while the decrease in type V collagen content is associated with the fish softening. The extent of proteolysis of the muscular fibers (Koohmaraie and Geesink 2006) along with the degree of alteration in the structural and other associated proteins estimates important eating quality trait termed as tenderness. It largely depends upon the extent of collagen crosslinks in the connective tissues and the contractile state of the skeletal muscle fibers (Lonergan et al. 2010). Structural changes like detachment of myofiber-to-myofiber connections and breaks in the myofiber-to-myocommata adhesions are largely responsible for the alterations in the fillet texture (Taylor et al. 2002). Muscle softening and fillet gaping caused textural changes in the Atlantic cod (*Gadus morhua* L.) fish (Kristoffersen et al. 2006). Textural and sensorial properties are the important quality affecting attributes closely linked to the fish freshness and post mortem degradation (Delbarre-Ladrat et al. 2006).

Textural degradation of surimi gels is attributed to the activity of the heat-stable alkaline proteinase (HAP). A poor elastic gel formation upon heating the white croaker meat paste to 60 °C was observed (Boye and Lanier 1988). HAP purified from the white croaker and Atlantic menhaden were reported as the cysteine proteinase showing optimum activity at 60 °C and pH 8.0. The presence of HAP activity is also reported in rainbow trout, sardine, carp, common mackerel, cod, herring and Atlantic salmon muscles (Stoknes and Rustad 1995). Cathepsins B, L and L-like possessing cysteine proteinase activities remain in the surimi even after washing and had myosin-degrading activity at high temperature and alkaline pH (Ho et al. 2000). Thereby, cathepsins are recognized as surimi gel softeners.

Serine protease inhibitors (SERPINs)

The seafood industry is interested in the economic natural inhibitors of proteases for keeping product quality. Protease inhibitors (PI) inhibit the process of the muscle protein degradation (Huang et al. 2009) and SERPINs are reported to be the most important ones among other classes of inhibitors (Zamora et al. 2005) i.e. cysteine, aspartic and metalloprotease inhibitors. SERPIN family includes over 3000 members having similar structures and are ubiquitous in origin, as have been identified in humans, plants, fungi, bacteria as well as in viruses too (Law et al. 2006). SERPINs have been recognized as the physiologically and therapeutically important class of proteins that have rapid and specific inhibitory ability (Elliott et al. 2000). Functionally, SERPINs irreversibly inhibit both the serine and the cysteine proteases (Olson and Gettins 2011) and are regarded as the cross-class inhibitors (Silverman et al. 2001). SERPINs can be utilized in preventing the fish softening, modori and textural damage.

Mechanism of inhibition

During inhibition, the reactive site of SERPIN encounters with the protease and inhibit its catalytic activity by entrapping it and forming a highly stable covalent intermediate, that resembles an acyl-protease complex. However, a small fraction of the SERPIN may also get consumed during the inhibition process. The inhibition reaction is extremely fast and the course for understanding the complexity of the inhibitory mechanism paved the way for the genetic engineering in SERPINs (Sulikowski et al. 2002). Protease–serpin reactions is not a simple 1:1 complex formation reaction rather it is a two-step process. The first step is the physical combining of the inhibitor to protease resulting in a non-covalent Michaelis complex and the subsequent irreversible step is the product forming. Its kinetic studies require a range of properties that depend upon measurements of three macroscopic parameters i.e. stoichiometry of inhibition (SI), complex breakdown rate constant (k_{brkdwn}) and inhibition rate constant (k_{inh}) (Schechter and Plotnick 2004).

The exogenous addition of protease inhibitors is capable of blocking the proteolytic activity in the muscle proteins (Xiong et al. 2012). These include both synthetic as well as natural ones viz. phenylmethylsulfonyl fluoride and p-toluenesulfonyl phenylalanine chloromethyl ketone (synthetic SERPINs); *N*-ethylmaleimide, iodoacetamide,

E-64 (synthetic cysteine PIs) and natural soybean trypsin inhibitor (SI) that inhibited the MHC degradation in the *walleye pollack* surimi (Hossain et al. 2001). The synthetic chemical inhibitors are not preferred in the food industry as they do not follow the necessary criterion for approval as the food additives. Other natural sources of muscle protease inhibitors from animal origin include mammalian blood plasma (1–2%) in the prevention of the degradation of surimi gels of Pacific whiting and Arrowtooth flounder. Egg white, beef plasma hydrolysate, and whey protein concentrate are other tested inhibitors of proteases. But these inhibitors from animal origin have their own negativities (e.g. off-flavor imparted by the egg white) that restrict their wide usage. Study of other protease inhibitors present in nature and their target proteases requires more investigations for used as food-grade inhibitors (Ouali et al. 2013).

Legumes seeds as a natural source of SERPINs

Legume seeds are consumed as an important staple food by humans. Besides having high nutritional value, they do contain certain biologically active compounds (Singh et al. 2017a, b, c). The search for natural inhibitors from plant sources leads to the isolation of a number of SERPINs from the seeds of various leguminous plants. They have been tested for their protease inhibitory activity and can be utilized in improving the fish meat quality during storage.

Trypsin inhibitor (TI) as modori preventive

Skeletal muscle proteases are responsible for modori phenomenon in surimi gels and researchers have tested a variety of legumes as inhibitors of modori. Soybean trypsin inhibitor (STI) was tested for its myosin heavy chain (MHC) degradation inhibitory activity in the washed and unwashed meat gels from three lizardfish species- Maeso or True (*Saurida undosquamis*), Wanieso (*Saurida wanieso*) and Tokageeso or shortfin (*Saurida elongate*) along with five chemical protease inhibitors viz. leupeptin, antipain, L-*trans*-epoxysuccinyl-leucylamido-4-guanidinobutane (E-64), *N*-ethylmaleimide (NEM) and ethylenediaminetetraacetic acid (EDTA)disodium salt (Suwansakornkul et al. 1993). The SDS-PAGE patterns of the myosin heavy chain (MHC) degradation from fish meat gels heated at 40 and 60 °C for 2 h revealed the concentration dependent proteolytic inhibitory effect of STI in all the three fish species. STI and leupeptin appeared as the potent inhibitors with high inhibitory effect. STI showed a relatively weak inhibitory effect in low concentration (400 µg/g) of meat gel, while at a raised concentration (4000 µg/g) of meat sol, the inhibitory effect was equivalent to leupeptin. E-64 showed inhibitory effect in two fish species i.e. Maeso and Tokageeso, while NEM remained confined to Maeso lizard fish only. However, EDTA (metalloproteinase inhibitor) didn't show any inhibitory effect on MHC degradation. Serine proteinase was identified as major and cysteine proteinase as minor factors responsible for the fish gel degradation. This study revealed the potential of STI as a food-grade protease inhibitor that can be exploited in controlling and preventing the surimi deterioration (Suwansakornkul et al. 1993).

In another study, STI inhibited proteolysis in surimi gels of tropical Tilapia (*Tilapia niloticus*) fish (Yongsawatdigul et al. 2000). The optimum temperature for the proteolytic activity in surimi was observed to be 65 °C as the activity ceases below 40 °C and above 70 °C. SDS-PAGE showed the complete disappearance of MHC band from the gel after 3 h of incubation without inhibitor at optimum temperature and prolonged incubation resulted in the appearance of 37 kDa band (hydrolytic protein product). Addition of the soybean trypsin inhibitor at a concentration of 50 µg/g completely inhibited the proteolysis in surimi gels. Leupeptin (cysteine and serine proteinases inhibitor) showed an inhibitory effect at the level of 500 µg/g, while the pepstatin (metalloproteinases inhibitor) did not show any inhibitory effect up to 100 µg/g. The significant inhibition by STI proved the involvement of serine proteases in the proteolytic process of surimi gels as modori inhibitor.

The partially purified trypsin inhibitor (TI) from dried seed powder of adzuki bean showed the inhibitory effect on proteolysis and gelling properties of threadfin bream (*Nemipterus bleekeri*) muscle (Klomklao and Benjakul 2015). The inhibitory effect of TI against sarcoplasmic proteinases and autolysis of threadfin bream mince and washed mince was concentration-dependent. TI at 30 g/l level sufficiently inhibited (76%) the total activity of sarcoplasmic proteinases indicating its potential as proteolytic inhibitor during surimi gelation. The autolysis (at 60 °C for 2 h) of mince and washed mince of threadfin bream was inhibited up to 78 and 82%, respectively in the presence of TI at the level of 3 g/100 g. SDS-PAGE pattern of the MHC confirmed the effectiveness of TI in proteolytic prevention. Increased TI concentration improved the band intensity in both mince and washed mince due to decreased degradation and more retained MHC contents. Breaking force and deformation of the kamoboko gel containing TI at a level of 3 g/100 g was increased (84.55 g and 29.12 g/100 g, respectively) as compared to the control gel without TI. This study revealed the effectiveness of TI in improving threadfin bream surimi gel properties.

Sarcoplasmic modori-inducing proteinases (Sp-60-MIP, Sp-50-MIP) extracted from the fillets of threadfin bream fish were effectively inhibited by partially purified protease inhibitors from pigeon pea (*Cajanus cajan*), cowpea (*Vigna*

unguiculata) and bambara groundnuts (*Voandzeia subterranea*) *var.* Hat Yai (HY) and *var.* typical (T) (Benjakul et al. 2000). For Sp-60-MIP, the highest inhibition (70%) was recorded for cowpea inhibitor (at the level of 500 units/ml), while pigeon pea inhibitor exhibited the maximum inhibition (20%) at a concentration of 5–10 units/ml. For Sp-50-MIP, the maximum inhibition achieved at the highest tested level was only 30–35%. The differences in specificity toward proteinases were presumed to be the reason behind the difference in inhibition efficiency of legume seed proteinase inhibitors. This presumption was further supported by a decreased extent of autolytic degradation and increased force and deformation in surimi gels by incorporation of proteinase inhibitors. The proteinase inhibitors of two bambara groundnut varieties and cowpea exhibited the highest inhibitory effect at the level of 10 kunits/g, while those of pigeon pea at same level showed the lowest inhibition to surimi degradation. The cowpea and bambara groundnut *var.* HY proteinase inhibitors at the level of 30 kunits/g resulted in an increase in gel force and deformation by 60% and 26%, respectively. SDS-PAGE of the surimi gels containing bambara groundnut and cowpea proteinase inhibitors has not revealed any noticeable change in the MHC band intensity. Thus, the results of the study indicated that the addition of proteinase inhibitors can effectively improve the strength of surimi gels.

In another study, the protein isolates from the mungbean (*Phaseolus aureus*) (MBPI) and black bean (*Phaseolus vulgaris* L.) (BBPI) were investigated for their protease inhibitory activity and effect on the gel strength from sardine (*Sardinella albella*) fish (Kudre et al. 2013). BBPI and MBPI at the level of 1 g/100 g were able to retain the MHC upon addition to surimi of the gel. The inhibitory activity against proteolysis of gels was found to be associated with the increased breaking force due to lower degradation. BBPI showed higher surimi gel strengthening effects as well as potent proteolytic inhibitory activity compared to MBPI (Table 1). The modori and kamaboko gels containing BBPI (1 g/100 g) showed increments in breaking force (90.1 and 24.6 g/100 g, respectively) and in deformation (35.6 and 11.5 g/100 g, respectively). The increased gel strength might be due to the improved myofibrillar protein–protein interaction leading to the stronger gel network. Thus, the addition of legume protein isolates may retard the endogenous protease-mediated proteolysis in sardine surimi. The impact of Bambara groundnut (*Vigna subterranean*) protein isolates (BGPI) on autolysis of the surimi gels of the threadfin bream (*Nemipterus bleekeri*) fish was tested (Oujifard et al. 2012). BGPI appeared as an alternative food-grade inhibitor that has the ability to improve the gelling properties without changing any sensorial characteristics (color, taste, elasticity, odor and flavor) of surimi. The appropriate amounts of BGPI responsible for increments in breaking force and deformation are 0–3 g/100 g for modori gel and 0.25 g/100 g for kamaboko gel. However, the addition of BGPI in higher amounts showed detrimental effects.

Water extracts of kidney bean (*Phaseolus vulgaris*) (10.1 mg/ml), pea (*Lathyrus sativus*) (6.0 mg/ml), chickpea (*Cicer arietinum*) (4.3 mg/ml), lentil (*Lens culinaris*) (3.5 mg/ml) and soybean (*Glycine max*) (11.6 mg/ml) seed flours were analyzed for their inhibitory effect on myosin degradation using Mexican flounder (*Cyclopsetta chittendeni*) and Atlantic croaker (*Micropogon undulatus*) fish muscle proteins (Ramírez et al. 2002). SDS-PAGE patterns of surimi gel treated with legume seed flour water extracts showed distinctive bands for myosin, whereas these bands were completely disappeared after 3 h in the non-treated surimi gels. This study reported reduced hydrolysis of myosin protein upon treatment with legume seed extracts. The differential inhibition of myosin degradation by legume seed flours observed was due to the variable protein concentrations in the water extracts. Legume seed extracts have the potential to reduce the proteolytic activity in fish flesh, prevent modori of surimi gels and partially inhibit the myosin degradation in meat. The legumes tested for softening inhibitory activity in fish are listed in Table 2. Legume seed flour can be used in maintaining the fish quality during storage. Among various tested legumes, soybean seed flour was the more effective candidate that can be used as a promising tool in the current scenario of fish preservation (Ramírez et al. 2002).

Table 1 Comparative textural properties of surimi gels containing protein isolate

Protein isolate	Breaking force g/100 g		Deformation increase g/100 g		References
	Modori	Kamaboko gels	Modori	Kamaboko gels	
BBPI (1 g/100 g)	90.1	24.6	35.6	11.5	Kudre et al. (2013)
MBPI(1 g/100 g)	76.8	22.4	28.1	11.6	Kudre et al. (2013)
BGPI (2 g/100 g)	109.43	–	47.92	–	Oujifard et al. (2012)
BGPI (0.25 g/100 g)	–	Increased	–	–	Oujifard et al. (2012)

BBPI black bean protein isolate, *MBPI* Mungbean protein isolate, *BGPI* Bambara groundnut protein isolate

Table 2 Legumes tested for inhibition of softening in fish

Active component	Legume	Tested component	Activity	Fish species	Reference
Seed extract	Palo blanco (*Lysiloma candida*) Palo fierro (*Olneya tesota*) Soybean (*Glycine max*) Gandul pigeon peas (*Cajanus cajans*) Palo verde (*Cercidium floridum* subsp. *peninsulare*) Garbanzo chickpea (*Cicer arietinum*)	Flesh juices	Flesh proteinase inhibition	Pacific whiting, parasitized and non-parasitized Merluza (*Merluccius productus*)	García-Carreño et al. (1996)
Meal	Soybean	Gastrointestinal crude digestive extracts	Gastrointestinal alkaline proteinase inhibition	Seabream (*Sparus aurata*) and Tilapia (*Oreochromis niloticus*)	López et al. (1999)
Defatted seed flour extracts (Water and NaCl)	Black cowpea, Soybean, Peanut, White cowpea, Mungbean	Viscera	Visceral proteinase inhibition	Threadfin bream (*Nemipterus bleekeri*)	Benjakul et al. (1999)
Trypsin inhibitor	Soybean	Surimi gels	Modori preventive	Tropical Tilapia (*Tilapia niloticus*)	Yongsawatdigul et al. (2000)
Trypsin inhibitor	Adzuki bean (*Vigna angularis*)	Surimi gel	Surimi autolysis prevention	Threadfin bream (*Nemipterus bleekeri*)	Klomklao and Benjakul (2015)
Defatted seed flour extracts (water, NaOH and NaCl)	Cowpea (*Vigna unguiculata* (*L.*) Wasp.), Pigeon pea (*Cajanus cajan* (*L.*) Millsp.), Bambara groundnut (*Voandzeia subferranea* (L.) Thou *var.* Typical (T) and *var.* Hat Yai (HY)	Fillet extract	Modori prevention	Threadfin bream (Nemipterus bleekeri)	Benjakul et al. (2000)
Seed flour Water extracts	Kidney bean, pea, chickpea, lentil and soybean	Surimi gel	Modori prevention	Atlantic croaker (*Micropogon undulatus*); Mexican flounder (*Cyclopsetta chittendeni*)	Ramírez et al. (2002)
Seed extracts	Soybean, red kidney bean, adzuki bean and bambara groundnut	Hepatopancreaic crude extract	Muscle softening prevention	Freshwater prawn (*Macrobrachium rosenbergii*)	Sriket et al. (2011a)
Protein isolate	Black bean and mung bean	Surimi gel	Kamaboko and modori prevention	Sardine (Sardinella albella)	Kudre et al. (2013)
Protein isolate	Soybean	Surimi gel	Modori prevention	Alaska pollock and common carp	Luo et al. (2004)
Protein isolate	Bambara groundnut	Surimi gel	Surimi autolysis prevention	Threadfin bream (*Nemipterus bleekeri*)	Oujifard et al. (2012)

Legume seed extracts as fish flesh juice inhibitors

The Pacific whiting (*Merluccius productus*) contains endogenous proteases in high amounts that damages myofibrillar proteins in muscles leading to the softening and unacceptable texture (Fowler and Park 2015). The seed extracts from various legume crops of California were tested for their protease inhibitory effect on Pacific whiting and parasitized as well as non-parasitized *merluza* fish flesh juices. Sorghum (*Sorghum bicolor*) and wheat (*Triticum aestivum*) were used as non-leguminous controls (poorest inhibitors). The proteinase specific activities estimated in the fish flesh showed significant differences and were observed in the order of Pacific whiting (2.736) > parasitized *merluza*

(0.708) > nonparasitized *merluza* (0.526). After the incubation period of 15 min at 90 °C, all legume seed extracts showed their proteinase inhibitory capability for commercially available trypsin, chymotrypsin (serine proteinases) and papain (cysteine proteinase). Seed extracts from six legume species viz. Palo blanco (*Lysiloma candida*), Palo fierro (*Olneya tesota*), soybean (*Glycine max*), Gandul pigeon peas (*Cajanus cajans*), Palo verde (*Cercidium floridum* subsp. *peninsulare*) and Garbanzo chickpea (*Cicer arietinum*) showed reduction in flesh juice proteinase activity in fishes by more than 50% on 30th day of incubation at 4 °C (García-Carreño et al. 1996). Seed extracts performed better in inhibiting the protease activity in unparasitized *merluza* in comparison to parasitized *merluza* and Pacific whiting. Palo blanco seed extract inhibited proteinase activity to 0% in *merluza* fish (both parasitized as well as nonparasitized). Soybean and Palo verde seed extracts totally inhibited proteinase activity to 0% in parasitized *merluza* only. Upon heating (up to 90 °C), the garbanzo, palo blanco and soybean seed extracts showed higher proteinase inhibition activity only for the Pacific whiting. The activity of the remaining three seed extracts was negatively affected by heat treatment. The overall order of inhibition observed was: non-parasitized *merluza* > parasitized *merluza* > Pacific whiting. Proteolytic activity in nonparasitized *merluza* was more effectively inhibited than in parasitized *merluza* and Pacific whiting. However, Pacific whiting enzymes were better inhibited by the seed extracts after heating. As the phenomenon of surimi gel disintegration is attributed to the proteases showing activity at > 50 °C, the garbanzo, palo blanco and soybean seeds showed a promising effect in preventing the modori. All the six selected legumes have utilizable potential in preventing fish softening.

Soybean meal as digestive extract inhibitor

Analysis of the soybean meal for the inhibitory activity on alkaline proteases present from the gastrointestinal tract was carried out using crude digestive extracts from Sea bream (*Sparus aurata*) and Tilapia (*Oreochromis niloticus*) fish (López et al. 1999). SDS-PAGE studies confirmed the presence of protease inhibitors in the soybean meal. The inhibition curves evidenced the exponential nature and diet-dependent physiological response to the inhibitors. Nearly, 35% reduction in the proteases activity was noticed in the Sea bream consuming feed (containing 30% soybean meal) equal to 1.5% of the fish weight. Both the Sea bream and Tilapia showed high sensitivity towards soybean since 40% inhibition was observed at very low levels (62.5 μg Unit of activity^{-1}) of the meal solution. However, protease inhibition response following the soybean meals was differential rather than uniform and was related to the sensitivity of fish species towards protease inhibitors. As digestive enzymes can induce extensive autolysis during storage or transportation, the above study highlights the potential of soybean meal in preventing the fish softening.

Legume seed flour extract as visceral proteinase inhibitor

Proteinase inhibitory activities (%) of water and NaCl extracts of defatted legume seed flour of five legume species (soybean, black cowpea, white cowpea, mungbean and peanut) were tested against threadfin bream fish visceral proteinases (Benjakul et al. 1999). Black cowpea and soybean showed high visceral proteinase inhibitory activities. The inhibitory activity was observed in the order of black cowpea (95.63) > soybean (84.11) > peanut (56.20) > white cowpea (56.13) > mungbean (55.14). Water and NaCl extracts of soybean and black cowpea showed high thermal stability with the remaining activity higher than 90% after 30 min of heating at 50 and 60 °C, respectively. The relative inhibitory activity of both legumes was reported to be the highest at neutral pH. Also over a broad pH range (3–10), the inhibitory activity remains higher than 90%, indicating their pH stability. However, the high salt (NaCl) contents showed a negative impact on the inhibitory activity of the black cowpea, which is probably supposed due to the inhibitor denaturation by high salt concentrations. Further investigation involving modori-inducing proteinases (MIPs) reported the inhibition of both Sp-50-MIP and Sp-60-MIP to a similar extent by soybean and black cowpea seed extracts. Seed extracts were found to be more effective inhibitors of sarcoplasmic MIPs (58–72% inhibition) than the myofibrillar MIPs (11–41% inhibition). The lesser susceptible of myofibrillar MIPs over sarcoplasmic MIPs was attributed to the limited exposure of proteinases to inhibitors due to their close association with muscles. This study proved the modori inhibiting potential of legume seed extracts which can be utilized in the surimi industry.

Legume seed extracts as an inhibitor of NAM and PSC degradation

The softening of freshwater prawn (*Macrobrachium rosenbergii*) during ice preservation is attributed to the internal serine protease activity mediated by trypsin-like proteases released from hepatopancreas. Seed extracts of adzuki bean, bambara groundnut, soybean and red kidney bean showed trypsin inhibitory activity against the hepatopancreatic crude extracts of freshwater prawn (Sriket et al. 2011a). The trypsin inhibitory activity was observed in the order of soybean > bambara groundnut > red kidney bean > adzuki bean. The inhibitory activity was increased by increasing the protein content up to the maximum concentration of 1 mg/ml in the hepatopancreas

crude extract and afterward remained constant. The maximum inhibition (60%) was observed for soybean and bambara groundnut. Highest inhibitory efficacy of soybean extract was revealed by the SDS–PAGE patterns of natural actomyosin (NAM) and pepsin soluble collagen (PSC) proteins (incubated for 1 h at 60 °C and 25 °C, respectively) with the hepatopancreatic crude extract. Soybean protein extract (0.5 mg/ml) and bambara groundnut protein extract (1.5 mg/ml) were proved effective in suppressing the hepatopancreas mediated NAM and PSC degradation. Both legumes can be used as food-grade inhibitors to retard degradation and to improve the shelf life of freshwater prawn during storage. The softening of freshwater prawn during ice preservation is attributed to the internal serine protease activity mediated by trypsin-like proteases released from hepatopancreas (Sriket et al. 2011a).

Conclusion

This review highlights the aspects of fish tenderness, the role of protease inhibitors and potential benefits of using legume seed flour or extracts in the fish meat industry. The fish industry recognizes the alteration in tenderness as an important manifestation of the protease activity. Muscle softening and degradation is the main encountered problems during processing and storage of fish. Serine proteases are responsible for the post-mortem tenderization which is generally considered as the most critical quality trait that affects consumer satisfaction and it needs a solution. Protease inhibitors that control the fish meat proteolysis are important candidates in this aspect. The application of food-grade protease inhibitors during storage appear as a novel means that can alleviate the problem of textural quality deterioration due to fish meat softening. Legume seed flour is a good source of protease inhibitors that can be used as an innovative trendsetter in the fish industry. Furthermore, studies are needed to prove the advantages of leguminous protease inhibitors in the meat industry. There are many promising possibilities exist in the process of understanding and solving the issues related to the meat tenderness using legume seed flour. Information provided in this review can be helpful in building consumer confidence towards the use of legume seed as a source of protease inhibitors in the fish industry.

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