CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Surimi, a refined form of mechanically deboned fish meat, has unique functionality including gel-forming ability and water-and oil-binding properties and can be used as a valuable ingredient or base component for a broad range of food products. However, proteolytic disintegration of surimi gels mostly occurring at a temperature close to 60°C (modori) causes an irreversible destruction of the gel structure of surimi (Niwa, 1992; An *et al*., 1996). This shows the detrimental effects on surimi quality by substantially lowering the gel strength giving brittle and nonelastic gels (Alvarez *et al*., 1999). Among numerous proteinases presented in muscle, endoproteolytic cysteine proteinases have the most serious effect on texture due to their thermostability and the ability to cleave the internal peptide bonds, while exopeptidases hydrolyze terminal peptide bonds (Kang and Lanier, 2000).

To alleviate the problems associated with protein degradation caused by the endogenous proteinases, proteinase inhibitors and other food-grade additives have been used to improve the physical properties of surimi gel (Reppond and Babbitt, 1993). Such inhibitors most commonly used include bovine plasma protein (BPP), potato powder (PP), egg white (EW), whey protein concentrate (WPC), and soy protein isolate (SPI) (Hamann *et al*., 1990; Wasson *et al*., 1992b; Morrissey *et al*., 1993; Akazawa *et al*., 1993; Park, 1994; Weerasinghe *et al*., 1996a, b). Among commercially available food grade protein additives, BPP was shown to be the most effective gel enhancer (Weerasinghe *et al*., 1996a). Despite their gel enhancing activity, these food-grade inhibitors have the limitation because they have a negative impact on surimi quality, especially discoloration and off flavor (Akazawa *et al*., 1993). Similarly, egg white is costly and has an undesirable egg-like odor at levels required for inhibition (Porter *et al*., 1993). Potato powder does not show any sensory limitations but causes some off-colors (Akazawa *et al*., 1993). Thus, the alternative food-grade proteinase inhibitors are needed in surimi suffering with gel softening such as Pacific whiting, arrowtooth flounder gel, etc.

Animal blood is a by-product of slaughterhouse and contains a number of proteins with high biological value. It can be used as a potential raw material in both feed and food ingredients owing to its good nutritional value and excellent functional properties (Tybor *et al*., 1975). However, blood has been used only in limited quantities for direct human consumption because of its intense color and characteristic taste. To maximize its use, the separations of blood into plasma and red cell fractions by centrifugation and ultrafiltration have been developed commercially (Cohn *et al*., 1946; Curling, 1980; Howell and Lawrie, 1983; Lee *et al*., 1987; Burnouf, 1995; Torres *et al*., 2002; Moure *et al*., 2003). Various proteinase inhibitors, including α_2-macroglobulin, proteinase inhibitor 1-4 (PI1- PI 4), postalbumin-1A, postalbumin-1B and kininogen have been found in bovine, porcine and human plasma (Harpel and Brower, 1983; Stratil *et al*., 1990; Lee *et al*., 2000a). The presence of these components in the plasma makes it a potential source of commercially valuable additives for surimi. However, uses of mammalian plasma protein, especially BPP have been suspected in contamination of Bovine Spongiform Encephalopathy (BSE) (Asher, 1999; Bradley, 1999). Chicken plasma is one of the potential novel additives for surimi processing due to the ease and abundance. Nevertheless, a little information regarding the use of chicken plasma, especially in surimi has been reported. The characterization and identification of proteinase inhibitor from chicken plasma would elucidate the mode of action and optimize the uses of chicken plasma in surimi.

1.2 Literature review

Proteolytic enzymes

Protease is a generic name given to those enzymes hydrolyzing the peptide bond in proteins. About 144 proteolytic enzymes of animal tissues have been classified (Barrett *et al*., 1980). These can be divided into two main families: the endopeptidases and the exopeptidases, which respectively cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus. The term "proteinase" is actually a synonym for endopeptidase (Barrett, 1995). Most of the proteinases are lysosomal and cytosolic enzymes, but some of them are in the sarcoplasm and associated

with myofibrils or external to the cell in macrophages. After disintegration of tissues, the enzymes are found mainly in the sarcoplasmic protein fraction (Jiang, 2000).

The standard method of classification proposed by the Enzyme Commission (EC) of the International Union of Biochemists (IUB) is based on the mode of catalysis. This divides the proteolytic enzymes into four groups: cysteine, serine, aspartic, and metallo, the name of each class being derived from a distinct catalytic group involved in the reaction. Regardless of the source, protease can be classified on the basis of their similarity to well-characterized proteases, such as trypsin-like, chymotrypsin-like, chymosin-like, or cathepsin-like (Barrett, 1995). They are also often classified according to their substrate specificity, the response to inhibitors, or by their mode of catalysis. Proteinases commonly found in fish muscle can be classified into three groups on the basis of their sensitivity to pH (Kang and Lanier, 2000) as follows:

Acid proteases (Lysosomal cathepsins)

The lysosomal system contains a large number (15-20) of proteinases within the membrane limited organelle and represents one of several pathways for intracellular degradation of muscle protein. The enzyme activities include exo- and endo-proteinases and, while they all share a common acidic pH optimum (3-4). Those enzymes exhibit the broad ranges of activity up to near neutrality (Zeece *et al*., 1992). There are 15 to 20 lysosomal cathepsins; however, only a few (principally endoproteinases) have been shown to be of significance to muscle protein degradation. The lysosomal proteinases found in muscle have been listed in Table 1 on the basis of some their characteristics.

Neutral and Ca^{2+}-activated proteases

Along with cathepsins and proteasome, calpain or calcium-dependent cysteine neutral proteases are myofibrillar proteases involved in the myofibrillar proteolysis that can result in post-mortem flesh tenderization. The complete disappearance of the Z-disk, titin, troponin-T and desmin, and weakening of the actin-myosin interaction induced by this enzyme was initially detected in muscle left in a Ca^{2+}(1mM)-containing solution (Davey and Gilbert, 1969; Koohmaraie, 1994). The enzyme is a ubiquitous protease and

exists either in the cell cytoplasm or associated with myofibrils, primarily in the region of the Z-disk (66% on Z-disk, 20% in I-band, and 14% in A-band) (Koohmaraie, 1994).

Table 1. Properties of some lysosomal proteases found in muscle.

Enzymes	Molecular weight (kDa)	Functional group	Optimal pH	Target proteins
A (endo-, exo-)	100	-OH	5.0-5.2	Less effect on intact proteins
B1 (endo-, exo-)	25	-SH	5.0	Myosin, actin, collagen
B2 (exo-)	47-52	-SH	5.5-6.0	Broad specificity
C (exo-)	200	-SH	5-6	Less effects on intact proteins
D (endo-)	42	COOH	3.0-4.5	Myosin, actin, titin, nebulin, M and C-proteins
E	90-100	COOH	2.0-3.5	Less effect on intact protein
H (endo-,amino-)	28	-SH	5.0	Actin, myosin
L (endo-)	24	-SH	3.0-6.5	Actin, myosin, collagen, α-actinin, troponin-T, I

Source: Kang and Lanier (2000).

Alkaline proteases

Alkaline proteases can easily interact with skeletal proteins, such as actin and myosin. Alkaline proteases are mostly heat stable and also become active at the neutral pH of fish meat paste, contributing to weaker elastic gel at 60°C than during incubation at 30-40°C or greater than 70°C (Makinodan *et al*., 1963). The content of alkaline proteinase in fish muscle may also be influenced by contamination of fish organ tissue such as kidney and liver, caused by poor cleaning technique (Su *et al*., 1981).

Role of proteolytic enzymes in fish muscle

Protease activity can be beneficial or deleterious in food production, processing and preparation. The post-mortem softening of fish and fish products due to

endogenous proteases is a serious problem. The lysosomal cathepsins and calpains are involved in the postmortem tenderization process and softening of fish gels (Jiang, 2000). Proteolytic enzymes involved in myofibrillar protein and gel degradation should meet the following three criteria (Koohmaraie, 1989):

- The enzyme must be located inside the skeletal muscle cells
- The enzyme must have the capacity to induce changes in myofibrils
- The enzyme must have access to myofibrils in the tissue

Effect of protease on fish muscle

Proteolysis of fish muscle during postmortem storage and processing has been associated with the endogenous muscle proteases, resulting in undesirable flavor and texture alterations (Sikorski and Kohakowski, 2000). The deterioration of fish muscle structure during storage is caused by the degradation of the connective tissue component of the muscle, leading to an ultrastructural changes and increase in collagen solubility (Kubota *et al*., 2001). It is generally accepted that the calcium-dependent proteinases (calpains) are responsible for myofibrillar and cytoskeletal proteins degradation. These changes induced degradation of titin and dystrophin, release of α-actinin from myofibrillar structure, and loss of Z-line integrity (Papa *et al*., 1996). The most detrimental effect of autolysis was found at 55°C during heating of Pacific whiting muscle (Morrissey *et al*., 1993; An *et al*., 1994a), and degradation of the myofibrillar proteins by cathepsin L was also most severe at this temperature (approximately 90% of myosin molecules were hydrolyzed within 5 min) (Chang-Lee *et al*., 1989)

Effect of proteinase on textural properties of fish gels

Gel weakening phenomenon is observed during cooking of surimi from some fish species. Temperature plays an important role in surimi gelation either by induction of network formation or by activation of endogenous enzymes. Proteolytic disintegration of surimi gels is enhanced at temperature above 50°C, in which the rapid and severe degradation of myofibrillar proteins, particularly myosin, takes place (Kang and Lanier, 2000). This disintegration has detrimental effects on surimi quality by lowering the gel strength and elasticity (An *et al*., 1996). The most active proteinases in fish muscle

that can soften the surimi gels vary with species, but are generally categorized into two major groups, i.e. cathepsins and heat-stable alkaline proteinases (Jiang, 2000).

High levels of cysteine proteinase activity including cathepsins B, H, L and L-like have been observed in Pacific whiting and arrowtooth flounder, chum salmon and mackerel (Yamashita *et al.*, 1990; Wasson *et al.*, 1992a; Lee *et al.*, 1993; An *et al.*, 1994). When the Pacific whiting muscle was incubated at 60°C for 30 min prior to cooking at 90°C, most myosin heavy chain (MHC) was degraded, and surimi did not form a gel with measurable gel strength (Morrissey *et al.*, 1993). In Pacific whiting surimi and arrowtooth flounder muscle, the reduction of gel strength or softening of muscle tissue was attributed to degradation of myofibrillar components like myosin, actin, beta-tropomyosin/troponin-T and collagen by cathepsin L which was maximally active at 55°C (An *et al.*, 1994; Visessanguan *et al.*, 2001).

Heat-stable alkaline proteinase has often been reported as responsible for textural degradation of surimi gels. It has been found in muscle from a large number of fish including rainbow trout, sardine, white croaker, carp, common mackerel, cod, herring, bigeye snapper and Atlantic salmon (Makinodan *et al.*, 1984; Stoknes *et al.*, 1993; Stoknes and Rustad, 1995; Benjakul *et al.*, 2003a). White croaker meat paste formed a poor elastic gel when heated around 60°C (Makinodan *et al.*, 1985). Proteinases associated with surimi gel softening were classified as modori-inducing proteinase (Toyohara *et al.*, 1990a; Ramos-Martiner *et al.*, 1999). Toyohara *et al.* (1990b) reported that MHC degradation was caused by a heat-stable serine proteinase termed the gel-degradation-inducing factor (GIF). GIF could degrade MHC at pH 7.0 in the presence of NaCl.

Protease inhibitor

An enzyme inhibitor is any substance that reduces the rate of an enzyme-catalyzed reaction (Whitaker, 1994). Protease inhibitors mimic the protein substrate by binding to the active site of the protease. Specific inhibitors are active-site-direct substances and combined with the catalytic or substrate-binding site of the enzyme to form a stable complex (Salvesen and Nagase, 1989). Nonspecific inhibitors are rare in nature, and the only one known is a plasma protein, α_2-macroglobulin (α_2M) (Barrett and

Starkey, 1973). Chelators that remove cations from metal-dependent proteases, and denaturants that alter catalytic sites are known as inactivators rather than inhibitors (Garcia-Carreno, 1996).

Inhibitors are divided into two types, the irreversible and the reversible inhibitors, based on kinetic considerations (Salvesen and Nagase, 1989). Irreversible inhibitors are generally low-molecular-weight site-directed compounds. The group of irreversible inhibitors includes all compounds that react with an enzyme to form kinetically stable covalent bonds. Most of the known irreversible inhibitors are synthetic substances that are used to determine the class of a protease. Reversible inhibitors are, in general, naturally occurring proteins and the enzyme activity is regenerated by displacement of the inhibitory molecule. Reversible inhibitors can be divided into three distinct types including competitive, non-competitive and uncompetitive inhibition, based on their effect on the slope and intercept of a reciprocal plot of observed initial rates versus initial substrate concentrations compared to the same reaction in the absence of inhibitors (Salvesen and Nagase, 1989).

Natural protease inhibitor

Inhibitors have been isolated from a variety of organisms including bacteria, animal and plants. Their sizes are also extremely variable from 50 residues (e. g bovine pancreatic trypsin inhibitor) to up to 400 residues (e. g alpha-1 protease inhibitor). They are strictly class-specific except proteins of α_2M which bind and inhibit most proteases through a molecular trap mechanism. Table 2 gives a list of organisms producing protease inhibitors.

Protease inhibitors commonly accumulate in high quantities in plant seeds, bird eggs and various body fluids. Protease inhibitors are also found in mammalian and marine animal blood plasma, where they account for more than 10% of total protein (Ylonen *et al*., 1999; 2002; Lee *et al*., 2000; Tahtinen *et al*., 2002). Furthermore, plant seeds such as legumes contain protease inhibitor which can be used to inhibit biological systems (Garcia-Carreno *et al*., 1996).

Table 2. Selected sources of protease inhibitors

Organism	Inhibitor	Characteristic	Affected enzyme
Microorganisms			
Actinomycetes	Leupeptine	Tripeptide	Calpain, cathepsin B,H, and L and chymotrypsin
Streptomyces testaceous	Pepstatin	Pentapeptide	Aspartic proteases: pepsin, cathepsins and HIV-1
Plants			
Soybean	NA	Protein	Trypsin
Lima bean	NA	Protein	Trypsin
Verterbrate organs and	**tissues**		
Plasma	α_2M	Protein	All classes
Pancreas	Aprotinin	Protein	Trypsin
Egg white	Ovomucoid	Protein	Serine proteases

Source: Garcia-Carreno and Hernandez-Cortes (2000).

Classification of protease inhibitors

Protease inhibitors can be broadly separated into two general categories based upon their spectrum of activity: the nonspecific protease inhibitors and the class-specific protease inhibitors. Nonspecific protease inhibitors are capable of inhibiting members of all 4 classes of proteases.

Cysteine protease inhibitors

These inhibitors act as a protective mechanism against cysteine proteases released into circulation after cell death. The cystatin superfamily contains three families of proteins that are related functionally as cysteine protease inhibitors and evolutionarily by their amino acid sequence identity. These inhibitors occur in all cells and body fluids of mammals and many lower organisms. The interaction of cystatins with cysteine peptidases is a reversible and tight-binding one at the active site, but without formation and cleavage of covalent bonds. The affinity of the cystatins to the lysosomal cysteine proteases is very

high. They do not react with serine or other types of proteases (Abrahamson *et al*., 1991). The cystatins are classified into three families as:

Family I: cystatin A, cystatin B. Synthesized without signal peptides; MW 11-12 kDa; contain no disulfide bonds; occur intracellularly in the cytosol.

Family II: cystatin C, D, S, SN, SA. Synthesized with signal peptides, MW 13-14 kDa, contain disulfide bonds; are secreted and present in the body fluids.

Family III: kininogens. Exist in several forms (L-kininogen, H-kininogen); MW 60-120 kDa; are glycoproteins; contain three cystatin domain, two of which are functional; occur mainly in blood plasma.

Serine protease inhibitors

Serine protease inhibitors comprise the largest super-family of the class-specific protease inhibitors. A feature of all the protease inhibitors in the serpin super-family is a particular peptide bond, located in a C-terminal domain, that is susceptible to attack by serine proteases (Carlson, 1996). These inhibitors are very abundant in mammalian plasma and plant cells and play a main role in many physiologic processes (Otlewski *et al*., 1999).

Antithrombin is a serine protease inhibitor involved in the coagulation cascade. Antithrombin III is one of the three plasma serpins that reacts 2-4 orders of magnitude more rapidly with target proteases in the presence of heparin. It is produced in the liver and endothelial cells and is responsible for 70% of the anticoagulant activity of normal plasma. Anti-thrombin forms a complex between the active site of thrombin and the reactive site of antithrombin. Thrombin or another protease binds to heparin, and brings the active site of the protease into close contact with the reactive site of antithrombin (Carlson, 1996).

Soybean trypsin inhibitors: Protease inhibitors that have been isolated from soybeans are of two types: the Kunitz trypsin inhibitor (TI) and the Bowman-Birk (BB) inhibitor. The first group has an MW between 20 and 25 kDa, with a specificity directed primarily toward trypsin. The inhibitor was shown to combine tightly with trypsin. The BB inhibitor is capable of inhibiting both trypsin and chymotrypsin at independent reactive sites. BB inhibitor has a stable conformation even after disulfide bonds are broken by heating (Kennedy, 1998).

Aspartic protease inhibitors

The best characterized aspartic proteases from mammals (pepsin, chymosin, cathepsin D and rennin) are all inhibited by pepstatin A. Aspartic proteinase inhibitors can be found in many sources such as potato, yeast, the nematode *Ascaris*, and squash (Garcia-Carreno and Hernandez-Cortes, 2000). The aspartic proteinase inhibitors from potato form a multigene family of at least 10 members (Ritonja *et al*., 1990). These inhibitors are similar to the soybean trypsin inhibitor family, which also possesses trypsin inhibitory activity. An inhibitor from squash phloem exudates (Christeller *et al*., 1998) has no similarity with any other known protein, which suggests that it belongs to a new inhibitor family. Squash, as yeast inhibitor, is an aspartic proteinase inhibitor that does not contain any disulfide bonds and there is no N-glycosylation site. Until recently, α_2-macroglobulin was thought to be the only major inhibitor of aspartic proteases (Thomas *et al*., 1989).

Metalloprotease inhibitors

Any substrate that complexes with and/or removes an essential cation from an apoenzyme will be an inhibitor of that enzyme (Whitaker, 1994). Most of the design of class specific inhibitors of metalloproteases has focused on attempts to chelate or bind the catalytic zinc atom. Synthetic inhibitors, therefore, commonly contain a negatively-charged moiety to which is attached a series of other groups designed to fit the specificity pockets of a particular protease (Whitaker, 1994).

An inhibitor can react directly with essential groups of the active site of the enzymes or with specific groups on the enzymes not involved in the active site per se (Whitaker, 1994). The most useful type of inhibitor in elucidation of reaction mechanisms is one that reacts with the active site of enzyme where substrate, cofactor, and/or activator are bound (Garcia-Carreno and Hernandez-Cortes, 2000). Table 3 shows a list of selected inhibitors, some properties, and mode of action.

Table 3. Mode of action of selected protease inhibitors

Inhibitors	Source	Characteristics
Inhibitor of factor Xa	Human	Specific, competitive, tight-binding ; two regions (residues 1-10 and 41-54) are responsible for the union to the factor Xa
Serine protease phosphonyl inhibitor	Synthetic	Irreversible, forms a stable enzyme derivative with the active site Ser-195
Serpins (Serine proteinase inhibitors)	Ubiquitous	44-110 kDa, undergoes conformational changes upon complex formation with enzyme that involves partial insertion of the reactive center loop into the-sheet of the inhibitor
Ovalbumin	Egg white	Although a serpin in nature, it is not a serine protease inhibitor because a charged residue, Arg, prevents the loop insertion into the sheet of enzyme
Aspartyl protease inhibitors	Synthetic	Transition state analogueĥydroxylphosphonate group in a P3-P1 framework increased the inhibitory activity (I_{50} = 10nM)

Source: Garcia-Carreno and Hernandez-Cortes (2000).

Plasma protease inhibitor

Plasma proteins have been classified in several different ways, e. g., by solubility, electrophoretic mobility, or functional class. A number of plasma proteins include enzymes, proenzymes or enzyme inhibitors (Fishman, 1960). Proteinase inhibitors normally found in plasma include α_2-macroglobulin, α_1-antichymotrypsin, α_2-antiplasmin, inter α-trypsin inhibitor, antithrombin III, C1 inhibitor and α_1-antitrypsin (Travis and Salvensen, 1983; Carlson, 1996; Kent and Drohan, 2001). Plasma protease inhibitors exhibit the varying functions (Table 4).

Table 4. Selected plasma protease inhibitors

Protein	Molecular weight (kDa)	Normal concentration (mg/dl)	Function
α_1-antitrypsin	54	2.5	Neutrophil elastase inhibitor
C1 inhibitor	104	0.24	Inhibitor of C1, factor XIIa and kallikrein
α_1-antichymotrysin	69	0.5	Inhibitor of neutrophil cathepsin G and mast cell chymse
α_2-antiplasmin	70	0.07	Plasmin
Antithrombin III	60	0.15	Thrombin and factor Xa inhibitor
Heparin Cofactor II	66	0.06	Thrombin inhibitor
Protein C inhibitor	57	0.004	Activated protein C
α_2-macroglubulin	725	2.7	Various
Inter-α-trypsin inhibitor	160	0.3	Unknown

Source: Carlson (1996).

Cysteine protease inhibitors in plasma

α_2- macroglobulin

α_2M is the first of two abundant, nonserpin protease inhibitors in plasma. It is very large protein and inhibits a broad spectrum of proteases. α_2M is composed of four subunits with a molecular weight of 180 kDa each and a total MW of 720 kDa (Harpel and Brower, 1983). Harpel and Brower (1983) also found that α_2M would also yield two protein bands at 125 kDa and 62 kDa when heated in the presence of SDS. It is able to bind serine, thiol, carboxyl, and metallo-proteinase (Barrett and Starkey, 1973) and each α_2M molecule can react with two endoproteinases at a time (Pochon and Bieth, 1983). It was first described as a proteinase inhibitor with the unique ability to inhibit proteinases with distinct trapping mechanism (Wu and Pizzo, 1999). The α_2M was

extracted from bovine, ovine, porcine and equine and it may be obtained by the recombinant DNA technology (Lorier and Aitken, 1991).

Kininogens

The kininogens are cysteine protease inhibitors found in plasma, belonging to the cystatin superfamily (Barrett *et al*., 1986). Kininogens found in plasma consist of two separate proteins, the low-molecular-weight kininogen (LMWK) with a MW of 50-78 kDa, and the high-molecular-weight kininogen (HMWK) with MW of 108-120 kDa (Kato *et al*., 1981; Turpeinen *et al*., 1981). Both kininogens were also found in porcine plasma which consisted of a single polypeptide chain (Mashiko *et al*., 1998; Lee *et al*., 2000a). Ornitho-kininogen, purified from chicken plasma protein, had the similar properties to mammalian HMWK (Kimura *et al*., 1987; Kos *et al*., 1992). The concentrations of LMWK and HMWK in human plasma were found to be 109-272 μg/ml and 69-116 μg/ml, respectively (Adam *et al*., 1985). LMWK is known to inhibit papain and cathepsin L (Salvesen *et al*., 1986).

Albumin

Bovine serum albumin, although not commonly known as a protease inhibitor, was found to be a nonspecific competitive inhibitor, which reduced enzyme activity in Pacific whiting surimi (Weerasinghe *et al*., 1996b). Albumin constitutes approximately 50% of the protein in plasma, with fibrinogen and α-, β-, and γ-globulins making up most of the remaining protein (White *et al*., 1973). Bovine plasma albumin is composed of a single peptide chain of about 580 residues (Peters, 1975) with MW ranging from 66.21 to 67 kDa (Putnum, 1975).

Application of protease inhibitors in food processing

Several protease inhibitors have been used to control proteolytic activity in muscle and also to improve the physical properties of surimi gels (Reppond and Babbitt, 1993). Commercial food-grade protease inhibitors used for surimi processing contain multiple active components (Table 5). It is possible that the enhanced gel strength by these

additives might be due to the combination of protease inhibition and gel enhancing factor (Weerasinghe *et al*., 1996b; Seymour *et al*., 1997). Wasson *et al*. (1992b) reported that plasma and egg white were able to increase gel strength when added at levels higher than necessary to prevent proteolytic activity. Higher punch test scores of Pacific whiting surimi gels was observed with the addition of whole plasma powder than whey protein concentrate or potato powder (Akazawa *et al*., 1993). The presence of proteinase inhibitors and TGase in BPP could enhance the gel network formation through promoting strong protein-protein interactions and reducing the proteolysis (Wasson *et al*., 1992b; Weerasinghe *et al*., 1996b). Saeki *et al*. (1995) reported that a marked decrease in breaking strength of heat-induced gel was effectively suppressed by addition of bovine plasma powder. This result was in accordance with Seymour *et al*. (1997) who reported that 1% BPP contributed to enhance gelation of Pacific whiting surimi by inhibition of fish protease and also by other gel-enhancing factors. Mackerel gel increased to 2-fold and 1.7-fold in breaking force and deformation, respectively with addition of 1% porcine plasma protein (PPP) (Lee *et al*., 2000b). Some limitations of these inhibitors are the off flavor and off color imparted to the final product (Wasson *et al*., 1992b; Akazawa *et al*., 1993; Seymour *et al*., 1997).

The increase in breaking force and deformation was observed when fraction I-S from porcine plasma was added into bigeye snapper surimi and incorporation of fraction IV-1 significantly increased the breaking force, deformation and water holding capacity of these surimi gels (Benjakul *et al*., 2001a, b). The addition of proteinase to actomyosin gels decreased in development rate and magnitude of gel modulus. However, lower gel modulus was observed with higher concentrations of PPP added (Visessanguan *et al*., 2000). Toyohara and Shimizu (1988) showed that the heat-stable proteases in threadfin bream were inhibited by soybean trypsin inhibitor (SBTI). The inhibitory effects of soybean trypsin were subsequently studied on oval-filefish meat gels that were softened by myofibrillar proteases (Toyohara *et al*., 1990b). They found that SBTI inhibited the breakdown of MHC at 50°C; however, it was not effective at 65°C. Lamb-Sutton (1995) found that purified α_2M and a crude plasma fraction containing this inhibited the inherent heat-stable proteolytic activity in surimi gels made from Atlantic menhaden, Pacific whiting, Alaska pollock and arrowtooth flounder as evidenced by SDS-PAGE analysis of protein integrity and gel mechanical properties by torsion testing.

Table 5. Commonly food grade inhibitors used in surimi processing

Protein additives	Active components	Functions	Application
BPP	Albumin, globulin, fibrinogen, α_2M, kininogen, FXIII	Gelling agent, protease inhibitor, gel enhancer	Pacific whiting, arrowtooth flounder, Alaska pollock, Atlantic menhaden
PPP	FXIII, albumin, postalbumin, α-PI, kininogen	Gel enhancer, proteinase inhibitor	Pacific whiting, mackerel bigeye snapper
Egg white protein	Ovalbumin, conalbumin, ovomucoi	serine protease inhibitor	Pacific whiting, arrowtooth flounder, Alaska pollock, Atlantic menhaden
Whey protein	β-lactoglobulin, α-lactoglobulin	cysteine protease inhibitor	Pacific whiting
Potato powder		numerous serine protease inhibitor and include cysteine protease inhibitor	Atlantic croaker, Pacific whiting, arrowtooth flounder, walley pollock
Legume seed extract and soy protein isolate	Oryzacystatin, globulin (2s, 7s, 11s and 15s)	Protease inhibitor (more serine protease inhibitor)	Atlantic croaker, Pacific whiting, arrowtooth flounder, Alaska pollock, Maxican flounder
Recombinant technology products	Cystatin	Protease inhibitor	Pacific whiting, mackerel, hairtail

Apart from the uses of inhibitors in surimi, inhibitors may be used as processing aids in all those food operation units affected by unwanted proteolysis. The fact that the control of proteolysis by inhibitors is so specific makes it a valuable tool in medicine, agri-culture and food technology. The presence of protease inhibitors in foods decreases the apparent nutritional quality of protein in the diet, by affecting the ability of

the body's digestive enzymes to degrade dietary protein, and thus limiting the intake of amino acids needed to construct new proteins (Garcia-Carreno, 1996). Gomez-Guillen *et al*. (2002) reported that the highest value of G′ of squid muscle was obtained when the sample was added with PMSF. However, the abrupt loss of shear moduli in chicken myofibril samples upon heating from 50 to 55°C was not prevented by protease inhibitors (Liu and Xiong, 1997). Lamb-Sutton (1995) investigated the diffusing solutions of such inhibitors into small chunks of arrowtooth flounder. Similar disappointing results for injection and soaking of fillets at commercial scale with plasma, egg white and other food-grade materials containing protease inhibitors were reported by McFarland (1990). Table 6 gives a list of potential uses of protease inhibitors in food processing.

Table 6. Potential uses of inhibitors in food industry

Seafood	Target enzyme or process
Underused marine resources	
Squid	Serine protease and cathepsin C reducing the gel forming ability of muscle
Caught or aquafarmed crustaceans	
Krill	Digestive enzymes-autolysis of tail muscle
Crayfish	
Langostilla	
Shrimp	
Fish processing	
Sardine	Cathepsins; reducing the gel-forming ability of muscle in surimi production
Haddock (Several species)	Limited collagen hydrolysis in descaling
Egg cells in roe and caviar production	Limited adhering connective tissue hydrolysis
Several inhibitor sources	Production of specialty marine enzymes; purification by affinity chromatography

Source: Garcia-Carreno and Hernandez-Cortes (2000).

Recently, Kang and Lanier (1999) successfully infused a recombinant cystatin into arrowtooth flounder muscle chunks by injection to achieve reduction of proteolytic activity during cooking, resulting in firming of the meat. Carvajal (2002) investigated the translational diffusion of enzyme inhibitors including α_2M, SBTI and cystatin through different subcellular compartments and intracellular architectures in the muscle cell. The result showed that low molecular weight protease inhibitors like cystatin can therefore be effectively diffused into intact fish muscle cells to minimize proteolytic activity and meat softening. Lamb-Sutton (1995) found that the crude plasma fraction enriched in α_2M also seemed to firm chunks of arrowtooth flounder during cooking at the optimum temperature for proteolytic activity, at a much lower total protein concentration in the diffusing solution than was required for a similar effect using beef plasma solution.

1.3 Objectives of study

1. To study the effect of chicken plasma protein on the properties of surimi gels.
2. To fractionate and characterize the cysteine proteinase inhibitor from chicken plasma and to investigate the efficacy of cysteine proteinase inhibitor fraction in inhibiting the autolysis and gelling properties of surimi.
3. To purify and characterize cysteine proteinase inhibitor from chicken plasma and to investigate its inhibitory effect on autolysis of myofibrillar proteins.