AN ABSTRACT OF THE THESIS OF

Ozlem Akpinar for the degree of Master of Science in Food Science and Technology presented on July 17, 1998. Title: Characterization of Recombinant Proteinase Inhibitors in Surimi Application

Abstract approved:

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High proteolytic activity Pacific whiting muscle causes hydrolysis of myofibrillar protein and lowers surimi gel quality. Although food grade proteinase inhibitors can be used to prevent autolytic activity in surimi, their usage is limited due to their adverse effects on the organoleptic quality of surimi. Cystatins however can be used as a specific cysteine proteinase inhibitor without adverse effects.

Recombinant cystatins were characterized for their inhibitory activity against cysteine proteinase and compared to egg white cystatin to develop a specific proteinase inhibitor to be used in Pacific whiting surimi. Soyacystatin expressed in *E. Coli* was purified with phenyl-Sepharose and DEAE 4.33 fold as a recombinant protein. Recombinant fish cystatin expressed in *E. coli* was also purified as a recombinant protein by affinity chromatography using Ni-NTA resin. Native egg white cystatin was purified 343 fold by using affinity chromatography on cm-papain-Sepharose. Fish cystatin, soyacystatin and egg white cystatin were tested for their inhibitory activity. The amount required to inhibit 50% activity of 2 μg papain was 0.245 μg and 0.455 μg for

soyacystatin and egg white cystatin, respectively. The amount of fish cystatin required to inhibit 90 % activity of papain was 3.57 μg while the amount of soyacystatin and egg white cystatin required to inhibit was 0.429 and 0.810 μg, respectively. For the characterization study, the fish cystatin was not tested because of low recovery in the purification process. Also, the harsh chemical treatment and lengthy period required to renature the protein made fish cystatin unfeasible for surimi application. Egg white cystatin and soyacystatin were tested against 400 ng of purified cathepsin L from Pacific whiting fillet. The amount of soyacystatin and egg white cystatin required to inhibit 50% activity of cathepsin L were 18.77 ng and 32.14 ng, respectively. The complex formation between papain and both cystatins was demonstrated by isoelectric focusing gel. When papain and cystatins formed a complex, the pI of the complex was resolved in between pIs of cystatins and papain, resulting in changes in pI from those of cystatins and papain. Both cystatins showed high stability at the wide pH range (pH 4-10), and soyacystatin was more sensitive at high temperature than egg white cystatin. Soyacystatin lost 80% of its activity at 60 ^{0}C by incubation for 30 min while egg white cystatin lost 70% of its activity by the same condition. Soyacystatin was tested for inhibition of autolytic activity in parasitized Pacific whiting fish fillet and Pacific whiting surimi. The results showed that 0.041% (w/w) soyacystatin inhibited almost 90% autolytic activity in Pacific whiting fish fillet. With Pacific whiting surimi, 75.57% autolytic activity was inhibited by 0.0052% (w/w) soyacystatin.

Characterization of Recombinant Proteinase Inhibitors in Surimi Application

by

Ozlem Akpinar

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented July 17, 1998
Commencement June 1999

Master of Science thesis of Ozlem Akpinar presented on July 17, 1998

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ACKNOWLEDGMENT

I would like to thank my major professor, Haejung An who provided constant encouragement and guidance during completion of this research. Also, I would like to thank Dr. Michael Morrissey for giving me an opportunity to work in the Seafood Lab; all the staff and students at the Seafood lab for making my life enjoyable and fun, and Dr. Hisashi Koiwa for providing the recombinant soyacystatin for this study and for his advice.

Finally, I would like to thank my parents and my friends for their unending support and encouragement.

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CHARACTERIZATION OF RECOMBINANT PROTEINASE INHIBITORS IN SURIMI APPLICATION

INTRODUCTION AND LITERATURE REVIEW

Pacific Whiting Surimi

Surimi is mainly composed of salt soluble myofibrillar proteins (An et al., 1996). Mechanically deboned fish muscle is washed and dewatered several times to produce surimi. With the washing procedure, the undesirable part of fish such as fat, blood, digestive enzymes, sarcoplasmic proteins and water soluble compounds are removed. Fresh fish contain 65% myofibrillar proteins and 18-20% sarcoplasmic proteins. After the washing step, 0.3% NaCl solution is added fish mince to facilitate the removal of water. At the end of the washing and dewatering step, the myofibrillar protein level typically goes up. To remove connective tissue and excess water, the washed mince is passed through a refiner and screw press and mixed with the cryoprotectants such as sugar, sorbitol and phosphate to stabilize the proteins during storage. To make surimi gel, myosin is dissolved by chopping with salt. The dissolved myosin combines with actin and forms actomyosin which gives the gelling characteristics of surimi. Before the resulting surimi paste sets to gel, it is extruded to a desired shape. The association and unfolding of denatured protein is responsible for the formation of the gel network at high temperatures (Lanier, 1986; Weerasinghe, 1995).

Surimi has been widely used as the main ingredient in seafood analogs such as artificial crab. Alaska pollock has been the species mostly used for surimi manufacturing It is difficult to meet the increased demand of surimi-based products solely with Alaska

pollock due to the maximized annual catch of Alaska pollock and its relatively higher price. Therefore, there has been a large effort to find new fish species for surimi production. Pacific whiting and arrowtooth flounder are some examples of new species which can be used for surimi production. Pacific whiting is the most abundant fishery resource off the U.S. Northwest coast. In the last few years, it has been successfully utilized in surimi production because of the large availability and the low price.

Proteolytic Degradation of Fish Muscle

Several fish species, such as, Pacific whiting, arrowtooth flounder, menhaden and white croaker suffer from postmortem softening. Fish muscle, after the death of the animal, becomes susceptible to autolysis by the endogenous muscle proteinases present. As a result of the hydrolysis, texture of the fish becomes soft and mushy. The degradation of myofibrillar proteins also causes adverse effects on surimi quality and lowers the gel strength (An et al., 1996). During heat setting of surimi based products, the formation of a three dimensional gel network is affected adversely by rapid breakdown of myofibrillar protein (Nishimoto et al., 1987). Temperatures above 50^{0}C, often occurring during cooking, accelerate enzymatic degradation of myofibrillar proteins, especially myosin. Cysteine endolytic proteinases have the most deleterious effect on the texture among all the proteinases present in muscle because of its thermostability and its ability to cleave internal peptide bonds producing shorter peptide chains. The proteinases that belong to this group are mostly caused by cathepsins and heat-stable alkaline proteinases (An et al, 1996; García-Carreño, 1996).

Parasite infection in fish muscle enhances the proteolytic activity and therefore hydrolysis of muscle and connective tissue proteins of the fish. The level of infection has been associated with varying proteolytic activity in fish, which causes variations in the gel strength of surimi. In Pacific whiting, *Kudoa paniformis* and *K. thyrsitis* have been identified as parasites (Morado and Sparks, 1986). During heating process, the most severe effects of autolysis in Pacific whiting muscle were observed (An et al, 1994a; Morrissey et al., 1993). The degradation of myofibrillar proteins by cysteine proteinase was also found to be highest at that temperature (An et al., 1994b). It was found that 90% of myosin molecules were hydrolyzed within 5 min.

Food Grade Proteinase Inhibitors

To utilize soft textured fish species, such as Pacific whiting, in surimi, it is important to control preoteinase activity during the processing. Bovine plasma proteins (BPP), egg white and potato powder (Hamann et al., 1990; Morrissey et al.,1993; Weerasinghe et al., 1996; Seymour et al., 1997) are the widely used food-grade proteinase inhibitors used. These inhibitors can inhibit autolytic activity as well as enhance the gelling effect in surimi. Use of food grade protease inhibitors however has been limited due to their adverse effects on organoleptic properties of surimi. For example, egg white used at the levels required for inhibition can cause off-color, off-flavor and an undesirable egg-like odor. Likewise, potato powder causes off-color in surimi. BPP causes off-color and off-flavor when it is used at concentrations above 1% (Weerasinghe, 1995). These adverse side effects of food grade proteinase inhibitors led us to find new proteinase

inhibitors for surimi processing. Therefore, we decided to use cysteine proteinase inhibitors as specific proteinase inhibitors for surimi processing.

Cathepsins

Cathepsins are known to act as endopeptidase, exopeptidase or both. Most cathepsins have acidic pH optima. Comparison of amino acid sequences shows that cathepsins B, H, L and S of mammalian lysosomes and cysteine proteinases of other higher plants belong to papain superfamily (Barrett, 1986).

Cathepsin L is as an endopeptidase, while cathepsin B can act as both endopeptidase and exopeptidase, and cathepsin H acts as an endoaminopeptidase (Kirschke and Barrett, 1987). Cathepsin B was purified from chum salmon (Yamashita and Konagaya, 1990a), mackerel (Matsumiya et al., 1989), and tilapia muscle (Sherekar et al., 1988). Also, cathepsin B was identified in the muscle from Pacific flounder (Geist and Crawford, 1974) and Pacific whiting (Erickson et al., 1983). Cathepsin H was identified as a major enzyme in mammalian muscle (Komionami et al., 1985), and it was isolated from chum salmon muscle (Yamashita and Konagaya, 1990b).

Cathepsin L

Cathepsin L (EC 3.4.22.15) is a cysteine proteinase very active in hydrolyzing myofibrillar proteins. It was found that the proteolytic activity of cathepsin L with myosin was 10 times greater than that of cathepsin B (Bird and Carter, 1980). Muscle cathepsin L was isolated from rabbit (Okitani et al., 1980), chum salmon (Yamashita and

Konagaya, 1990c), mackerel (Ueno et al., 1988) and Pacific whiting (Seymour et al., 1994). Although cathepsin B is the most active cysteine proteinase in Pacific whiting fillets, cathepsin L is the major source of proteolytic activity in Pacific whiting surimi. Cathepsin B and H and other proteinases are removed during the processing of surimi by washing but cathepsin L is not completely removed by the washing step (An et al., 1994b).

Cathepsin L was purified from Pacific whiting muscle and characterized by An et al. (1994b) and Seymour et al. (1994). It was found that the purified cathepsin L had a optimum temperature of 55°C and a optimum pH of 5.25-5.5. It is made of a single peptide with a molecular weight of 28,800 (An et al., 1994b, 1996) and has an isoelectric point of 4.91 (Seymour et al., 1994). The amino acid composition of the purified cathepsin L showed a high proportion of hydrophobic amino acids (Nickel, 1996). Also myosin has high affinity to hydrophobic binding sites (Borejdo, 1983). The high proteolytic activity of Pacific whiting surimi may come from the high hydrophobic interaction between cathepsin L and myofibrillar proteins. Purified cathepsin L was sequenced for N-terminus, and 23 of 25 amino acid residues were identified (Nickel, 1996). When the sequence was compared with N-terminal sequence of the member of cysteine proteinase, it was found that this sequence was 65 % identical with human (Mason et al., 1986) and mouse cathepsin L (Dufour et al., 1987).

Cystatin

Cystatins are found in animal tissues, human biological fluids, egg white and plants (Barrett et al., 1986) . The proteins of cystatin superfamily are grouped into four different families based on their molecular structure. Cystatin family I, stefin, are known to lack disulfide bonds. Their molecular weight is about 11,000 and they have been isolated from human and rat tissues (Barrett et al., 1986; Rawlings and Barrett, 1990). Cystatins family II contains 2 disulfide bridges. Examples of cystatin family II are chicken cystatin (Barrett, 1981), cystatin C (Barrett et al., 1984) and cystatin S (Isemura et al., 1984). Cystatin C which is found in human cerebrospinal fluid, urine from patients with renal failure, ascites, pleural fluids and fish. Cystatin S is a low molecular weight acidic protein and was isolated from human saliva S (Isemura et al., 1984). Cystatin family III, also called kininogens, have a large molecular weight, M_r 70,000 (Gounaris et al., 1984). They are found in rat skin (Barrett et al., 1986). Cystatins family 4, also called phytocystatins, were recently discovered and found in plants (Turk et al., 1997). Although there are four different type of cysteine proteinases, their physiological functions are common in all cystatin families. Cystatins are involved in the regulation of protein turnover and the pathological process resulting in disease mitigation such as viral replication, bacterial infection, and tumor metastasis (Henskens et al., 1996).

Inhibition mechanism of cystatin

Cystatin forms a reversible, tight complex with cysteine proteinase at an equimolar ratio (Bjork et al., 1989). Cystatins inhibit cysteine proteinases by making the

reactive site of the enzyme inaccessible to substrates and to the thiol group reagents (Nicklin and Barrett, 1984; Bjork et al., 1989). On the basis of chicken egg white cystatin structure, it was suggested that there are three regions which are responsible for the contact in the interaction with protease: the N-terminal 11 amino acid residues and two hairpin loops that connect the strands of B sheet (Turk et al., 1997). The peptide segment from Gln-53 to Gln-57 (QVVAG region) folds into first B-hairpin loop which is present between the N-terminal segments and second B-hairpin loop that contains Pro-Trp residues. The complex of cystatin and cysteine proteinase shows steric fit between these three regions of cystatin and the active site cleft of the enzyme (Turk and Wolfram, 1991; Turk et al., 1997).

Egg white cystatin

Egg white cystatin is the first characterized low molecular weight, tight-binding proteinase inhibitor of plant cysteine proteinases, i.e., papain and ficin (Anastasi et al., 1983; Fossum and Whitaker, 1968; Nicklin and Barrett, 1984; Schwabe et al., 1984; Turk et al., 1983). It was first isolated in small quantities from egg white by Fossum and Whitaker (1968) and was referred to as a ficin and papain inhibitor. The name "cystatin" for this protein was proposed by Barrett (1981), because out of all proteinase inhibitors, it has distinguishing ability to form a complex with cysteine proteinase even after its catalytic sites have been inactivated by bulky active-site-directed reagents. It also inhibits cathepsin B, dipeptidyl peptidase (cathepsin C), cathepsin L and H. Egg white cystatin belongs to cystatin family II, because it contains two disulfide bonds. It is composed of

115 amino acid residues, and its molecular weight is M_r 13,143 (Turk and Wolfram, 1991; Nicklin and Barrett, 1984). Egg white cystatin was isolated with the mixture of two major proteins having the same molecular weight but different isoelectric points (Anastasi et al., 1983). One form, non-phosphorylated form, has pI of 6.5 and the other form, phosphorylated form has pI of 5.6 (Laber et al., 1989; Turk and Wolfram, 1991). Although chicken cystatin is not present at a high level in egg white (0.05%), it has been detected in serums of male and female chickens and chicken muscle cells (Anastasi et al., 1983). Anastasi et al. (1983) used cm-papain-Sepharose column and chromafocusing to purify egg white cystatin. Later, egg white cystatin was cloned and expressed in *E.coli* (Turk et al., 1983; Schwabe et al., 1984). It was found that the inhibitory activity of recombinant and native egg white cystatins were similar (Auerswald et al., 1991). It has been crystallized (Bode et al., 1985), and its three dimensional structure was defined with X-ray diffraction methods (Bode et al., 1988). Complex formation with papain was elucidated based on the crystal structure of egg white cystatin (Bode et al., 1988).

Fish cystatin C

Fish cystatin C belongs to family II which contains disulfide bonds. Cystatin was found to be widely distributed in fish tissues. During spawning migration of salmon, cystatin activity in most tissues seems to be low. The cystatin activity is closely related to the physiological conditions of fish, such as sexual maturation or starvation during the spawning migration of fish (Yamashita and Konagaya, 1991). Salmon flesh suffers from extensive hydrolysis of muscle proteins during spawning migration. During this period

the cathepsin L activity in the salmon muscle was significantly increased (Ando et al., 1985). The increased cathepsin L activity is thought to be due to the decreased activity of cystatin in fish. It was shown that a large proportion of cathepsin L-like protease occurred as a complex with endogenous protease inhibitors (Benjakul et al., 1996). Based on the dissociation characteristics and the molecular weight, this endogenous proteinase inhibitors was thought to be cystatin (An et al., 1995). Yamashita and Konagaya (1992a) found cathepsin L-inhibitor complex in salmon muscle. They found that in the later study the complex consisted of 37 kDa-cathepsin L and 15 kDa-endogenous cysteine proteinase inhibitor, cystatin (Yamahita and Konagaya, 1992b). Cystatin from the eggs of chum salmon was purified and its complete amino acid sequences were determined (Yamashita and Konagaya, 1996). Cystatin cDNA was isolated from salmon pituitary gland and its amino acid sequence was determined. Amino acid sequences of salmon cystatin showed that it had 111 amino acid residues with two disulfide linkages. This result showed that salmon cystatin belongs to cystatin family II (Koide and Noso, 1994). Cystatin cDNA was isolated from rainbow trout liver and the amino acid sequence was determined (Li, 1997). It was shown that rainbow trout cystatin has four cysteine residues forming two intramolecular disulfide bond. The rainbow trout cystatin showed high sequence identities with salmon (Koide and Noso, 1994) and chicken cystatin (Colella et al., 1989).

Phytocystatin

Phytocystatin shows a wide inhibition spectrum against cysteine proteinases from plant and animal origin. Abe et al. (1994) reported that corn cystatin inhibited various cysteine proteinase, including cathepsins H and L and papain. It also weakly inhibits cathepsin B. One function of phytocystatin is to regulate the action of cysteine proteinase in seeds during ripening and germination process. Another function is to recognize and inhibit exogenous proteinases such as digestive enzymes of pests (Irie et al., 1996).

Oryzacystatin from rice seeds was the first plant cystatin isolated and investigated for its inhibitory activity and the cDNA sequence (Abe and Arai, 1985; Abe et al., 1987). Oryzacystatin was first isolated from rice seeds (Abe et al., 1987). Later, oryzacystatin was identified in 11 major rice varieties grown in California (Izquierdo-Pulido, 1994). The subsequent study showed that rice seeds had another cysteine proteinase inhibitor, called oryzacystatin II, based on the structure and enzyme specificity of oryzacystatin I (Kondo et al., 1990). Kudo et al. (1998) purified and crystallized oryzacystatin I which was overexpressed in *E. coli*. Oryzacystatin does not have a disulfide bond; thus, they are more similar to cystatins from family I. Their amino acid sequence however are more similar to cystatins from family II. Moreover, the gene structure of oryzacystatin is different from cystatins of the other family. Cystatin from a plant source, therefore, is classified as another family and referred to as phytocystatin family (Abe et al., 1992; Turk et al., 1997).

Brzin et al. (1990) isolated and determined partial amino acid sequence of the soyacystatin. Hines et al. (1991) purified the soyacystatin from soybean seeds and partially characterized it. Three cystatin clones from the soybean embryo library and

those encoding monodomain soyacystatins were isolated (Botella et al., 1996; Zhao et al., 1996). It was found that soyacystatins are a reversible noncompetitive inhibitor of papain (Zhao et al., 1996). Both native soyacystatins and recombinant soyacystatins have almost the same inhibitory activity (Misaka et al., 1996; Hines et al., 1991). The soyacystatins inhibited the larval growth and development in feeding bioassay (Koiwa et al., 1998).

Plants protect themselves against pest's attack by producing proteinase inhibitors (Koiwa et al., 1997). Proteinase inhibitors produced by plants inhibit activity of pest's proteinases and, subsequently, they affect nutrient assimilation and other essential metabolic processes (Koiwa et al., 1997). It was shown that when the insect *Acanthoscelides abtectus* was fed with an artificial bean-seed system containing a cysteine proteinase inhibitor, the development of larvae was delayed and the mortality of larvae was increased (Hines et al., 1991). Chen et al. (1992) also reported that oryzacystatin inhibited 80% activity of proteases in the rice weevil gut. Corn cystatin prepared from transgenic rice plants showed inhibitory activity against gut proteases of the insect pest (Irie et al., 1996).

Papaya cystatin was isolated and expressed in *E.coli* from papaya leaves (Song et al., 1995). From the fruit of avocado, a cysteine proteinase inhibitor was purified and its complete amino acid structure was determined (Kimura et al., 1995). Corn cystatin I was expressed in *E. coli* and purified (Abe et al., 1994). Cystatin from pineapple stems was purified and the complete amino acid sequence was determined (Lenarcic et al., 1992). Other phytocystatins were isolated form plant sources: potato (Rodis and Hoff , 1984; Brzin et al., 1988; Rowan et al., 1990), cowpeas (Rele et al., 1980), mung beans

(Baumgarter and Chrispeels, 1976), Brazillian beans (Olivia et al., 1988), seeds of the luguminous Bahiminia tree (Goldstein et al., 1973).

Application of Cystatin in Foods

Legume seeds inhibit digestive enzymes effectively due to the high content of proteinase inhibitors. Legume seed extracts were shown to inhibit both serine and cysteine proteinase which were responsible for the proteolytic degradation of fish muscle. It was reported that several seed extracts showed promising results as a proteinase inhibitor in the surimi production (García-Carreño, 1996). Izquierdo-Pulido et al. (1994) reported that cystatin isolated from rice was inhibitory against heat activated arrowtooth flounder proteinase.

Specific cysteine proteinase inhibitors reduced the activity in the muscle of Pacific whiting. E-64, a cysteine proteinase inhibitor, completely inhibited the activity in the Pacific whiting muscle (García-Carreño et al., 1998). Commercial egg white cystatin and E-64 showed reduction of the protease activity into a negligible level in Pacific whiting surimi (An et al., 1994a). These results led us to use a specific cysteine proteinase inhibitor in surimi to control proteolysis during processing.

Recombinant DNA technology provides methods to manipulate thousands of the nucleotides of the gene to change the properties of the expressed protein (Whitaker, 1996). Molecular cloning is a powerful tool to produce pure materials with high yield for chemical, biological and genetic analyses. It starts with isolating a DNA fragment containing the gene of interest which is linked to another DNA molecule, such as a

plasmid that is capable of directing its own replication (Mathews and Van Holde, 1996). The molecule is then introduced into a bacterial cell and the recombinant DNA molecule replicates along with the bacteria cell as the bacterium grows. Therefore, we investigated the use of a recombinant cystatin as a specific cysteine proteinase inhibitor and compared the efficiency of proteinase inhibition in the production of surimi.

Research Objectives

The objectives of this study are to:

1. Purify recombinant fish and soyacystatin expressed in *E. coli*,
2. Characterize biochemical properties of recombinant fish cystatin and soyacystatin and compare the inhibition efficiency to that of purified egg white cystatin against papain, purified Pacific whiting cathepsin L and Pacific whiting surimi.

The long-term goal of this research is to find an effective inhibitor that is specific and cost effective in inhibiting autolytic activity in Pacific whiting surimi.

MATERIALS AND METHODS

Materials

Kanamycin, ampicillin, isopropyl β-D-thiogalactopyronoside (IPTG), papain, Sepharose 6B, Brij 35 (30% w/v), glycerol, benzoly-DL-arginine-*p*-nitroanilide (BAPNA), N-benzoyl-L arginine-2-naphthylamide (BANA), N-carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin (Z-Phe-Arg-Nmec), L-trans-epoxysuccinylleucylamido (4-guadino) butane (E-64), DMSO, reduced glutathione, oxidized glutathione, β-mercaptoethanol (β–ME), *p*-dimethylaminocinnamaldehyde, trizma base, tricine, ammonium sulfate (AS), dithioerythritol, bovine serum albumin (BSA), L-tyrosine, low molecular weight standards including aprotinin (6,500), α-lactalbumin (14,200), trypsin inhibitor (20,000), trypsinogen (24,000), carbonic anhydrase (29,000), gylceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000) and albumin (66,000), were purchased from Sigma Chem. Co., St. Louis, MO. Iodoacetic acid was obtained from Calbiochem (San Diego, CA). Fast Garnet GBC base (4-amino-2,3-dimethylazobenzene) was obtained from Aldrich Chem. Co., Milwaukee, WI. Phenly-Sepharose 6 fast flow, DEAE Sephorose fast flow, butyl-Sepharose 4B, broad range of pI standards including trypsinogen (pI-9.3), lentil lectin-basic band (pI-8.65), lentil lectin-middle band (pI-8.45), lentil lectin-acidic band (pI-8.15), myoglobin-basic band (pI-7.35), myoglobin-acidic band (pI-6.85), human carbonic anhydrase B (pI-6.55), bovine carbonic anhydrase (pI-5.85), β-lactoglobulin A (pI-5.20), soybean trypsin inhibitor (pI-4.55) and amyloglucosidase (pI-3.50), were puchased from Pharmacia, Piscataway, NJ. Ni-NTA

was obtained from Qiagen Inc., Santa Clarita, CA. Premade agarose gel for isoelectric focusing was puchased from FMC Corp., Rockland, ME. Sodium caseinate and Mersalyl acid was purchased from U.S. Biochemical Corp., Cleveland, OH.

Ampicillin and IPTG solution were prepared as 1 M stock solution in water and sterilized by filtration through 0.2 μm sterile Acrodisc (Gelman Sciences, Ann Arbor, MI). The stock solutions were stored -20 ^{0}C until used. The stock solution of synthetic substrates and E-64 were prepared in DMSO and stored -20 ^{0}C until used. Surimi samples without any inhibitor were obtained form local commercial processor and was packaged in individual plastic bags and kept in storage at -18 ^{0}C.

Methods

Purification of soyacystatin

Growth of recombinant cells

The soluble recombinant proteins were constructed and overexpressed by Hisashi Koiwa (Purdue Univ. West Lafayette, IN). The cDNA had been inserted into pET28a (Novagen, WI) to produce pETNM$^{8\text{-}103}$. pETNM$^{8\text{-}103}$ was introduced into BL21 (DE3) cells for recombinant protein expression (Koiwa et al., 1998). The recombinant cells were grown in small scale in 5 mL LB broth with 50 μg/L of kanamycin overnight at 37 ^{0}C with vigorous shaking. The following day it was inoculated into a large media (250 mL LB broth with 50 μg/L of kanamycin and allowed to grow until A_{600} reached to 0.6 (generally 3-4 hours after inoculation into a large culture). Finally, it was induced

with 0.4 mM IPTG (final concentration). The recombinant proteins accumulated in cells grown for 16 hr at room temperature, and the cells were harvested by centrifuging at 4,000*xg* for 30 min using a Sorvall Model SS-34 rotor refrigerated centrifuge (DuPont Co., Newtown, CT).

Purification of soyacystatin with phenyl-Sepharose and DEAE

Harvested cells were sonicated using Sonicor, Model UP-400 with ultrasonic probe (Copiague, NY), in 10 mL of 10-fold diluted McIlvaine's buffer, pH 7, in the ice, until clear solution was obtained using. Sonicated cell extract was centrifuged at 7,000*xg* for 10 min. The resulting supernatant was precipitated with 70% saturated AS. The precipitated protein was dissolved in 10 mL of 10 mM potassium phosphate, pH 6 and centrifuged at 7,000*xg* for 10 min to remove the debris. To the supernatant, 20 % saturated AS was added and centrifuged at 7,000*xg* for 10 min. The final supernatant was loaded onto phenyl-Sepharose column and equilibrated with 20 mM potassium phosphate, pH 6, containing 20% saturated AS. While the sample was eluted, the A_{280} value of each fraction was monitored. Elution was started with 15 % saturated AS in 20 mM potassium phosphate buffer. When A_{280} reading of the fraction started to decrease, the elution buffer was shifted to 10 % saturated AS in the same buffer.

Fractions were analyzed for protein concentration and cystatin band on SDS-Tricine PAGE. The fractions which had a visible cystatin band were combined. Into the combined fractions, 80 % saturated AS was added, stirred overnight at 4^0C and centrifuged at 7,000*xg* for 10 min. The pellet was suspended in 10 mM Tris, pH 8.8 and dialyzed against 10 mM Tris, pH 8.8. The dialyzed sample was loaded in DEAE column

equilibrated with 10 mM Tris, pH 8.8. After loading the sample, the column was washed with 10 mM Tris, pH 8.8, overnight and eluted with the linear gradient of 0-0.4 M NaCl in10 mM Tris, pH 8.8. The fractions which had a cystatin band on SDS-tricine PAGE, were combined. Combined fractions were dialyzed against 50 mM sodium phosphate, pH 6 (Koiwa et al., 1998). The activity of combined fractions were analyzed for inhibitory activity as in section " inhibition assay against papain".

Purification of egg white cystatin

Preparation of cm-papain-Sepharose

Cm-papain-Sepharose column was prepared according to the method of Axen and Ernback (1971). Papain (100 mg) was activated with 2 mM dithioerythritol and 1 mM disodium EDTA in10 mL of 0.1 M sodium phosphate, pH 6, for 10 min at 20 ^{0}C and allowed to react with 10 mM iodoacetic acid. For activation of Sepharose resin, 5 mL water and 4 mL CNBr from 25 mg/mL stock solution of CNBr were mixed with 100 mg of Sepharose 6B resin. The pH of the activated Sepharose resin was kept at 10-11 with 2 N NaOH for 8 min. After the pH treatment, the gel was washed with cold 500 mL of 0.1 M $NaHCO_3$, pH 9.0. Activated papain solution was stirred with the Sepharose 6B overnight at room temperature for coupling. The resin was washed with 500 mL of 0.01 sodium acetate, pH 4.1; 400 mL of 0.1 M sodium phosphate, pH 7.6, containing 1 M NaCl; 200 mL of 0.1 M sodium phosphate, pH 7.6, containing 15 g/L glycine; 400 mL of 0.1 M sodium phosphate pH 7.6 containing 1 M NaCl; and finally 500 mL of 0.01 sodium acetate, pH 4.1.

Affinity chromatography for purification of egg white cystatin

Egg white cystatin was purified from twelve eggs according to Anastasi et al. (1983). The egg white was blended with equal volume of 0.25% (w/v) NaCl. The pH of the solution was adjusted to 6-6.5 with 5 M sodium formate buffer, pH 3. To remove ovomucin from the egg white the solution was centrifuged at 2,100*xg* for 30 min.

Cm-papain-Sepharose, 25 mL, was equilibrated with 50 mM phosphate buffer, pH 6.5 containing 0.5 M NaCl and 0.1 % Brij. The centrifuged egg white solution was stirred with the equilibrated cm-papain-Sepharose overnight at 4 ^{0}C. The resin was washed with 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 0.1 % Brij until the A_{280} was less than 0.05. The cm-papain-Sepharose was packed into a column and washed with 2 bed volumes of 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 10% (v/v) glycerol. The bound protein was eluted with 50 mM phosphate buffer, pH 11.5, containing 0.5 M NaCl and 10% (v/v) glycerol. Fractions, 2 mL, showing inhibitory activity in BAPNA assay were combined and the pH was adjusted to 7.4 with 5 M sodium formate buffer, pH 3.

Cystatin inhibitory activity assay using papain-BAPNA system

The assay buffer was composed of 200 mM K_2HPO_4, 200 mM KH_2PO_4, 4 mM EDTA and 4 mM dithioerythritol. For a substrate, 50 mM BAPNA in DMSO was used. Papain solution was prepared at the concentration of 3 mg/mL in water. Assay buffer, 0.5 mL, was mixed with 0.1 mL of papain and 0-0.1 mL inhibitor solutions. The final volume was adjusted to 1.25 mL with water. This mixture was incubated at room temperature for 10 min and the reaction was initiated by the addition of 0.05 mL substrate solution. The

reaction was carried out for 10 min using Beckman DU 640 spectrophotometer (Salt Lake City, UT). The reaction rate of residual activity of papain was determined by monitoring the absorbance change at 410 nm due to *p*-nitroaniline released during 10 min reaction period. The inhibitory activity was measured by change in the reaction rate. One unit of activity was defined as the change in absorbance of 1.0 per minute (Barrett, 1981).

Purification of recombinant fish cystatin

Growth of recombinant fish cystatin cells

The rainbow trout cystatin cDNA inserted into pRSET-B vector under T7 promoter was expressed as a fusion protein with 6xHis tag in *E. coli* by Fugen Li (1998) (OSU, Corvallis, OR). The recombinant cells were grown in 10 mL LB broth with 100 μg/mL of ampicillin overnight at 37 ^{0}C with vigorous shaking. The following day, the cell was inoculated into a large culture (500 mL LB broth with 100 μg/mL of ampicillin) and grown at 37 ^{0}C until A_{600} reached 0.7-0.9. Then the recombinant cells were induced with 1 mM IPTG (final concentration). After 3 hours of induction, the cells were harvested by centrifugation at 4,000*xg* for 30 min at 4 ^{0}C using a Sorvall Model SS-34 rotor refrigerated centrifuge (DuPont Co., Newtown, CT).

Purification of recombinant fish cystatin under denaturing condition

The cells expressing recombinant fish cystatin were suspended in 6 M GuHCl, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0 (Buffer A) at 5 mL per gram of wet weight and stirred for 1 hr at room temperature. The cell lysate was centrifuged for 15 min at 4 ^{0}C at 10,000*xg* and the supernatant was collected. Ni-NTA resin, 4 mL, was

packed into the column and equilibrated with buffer A. The centrifuged cell lysate was loaded on the column at the rate of 0.3-0.4 mL/min. After loading the sample, the column was washed with 10 column volumes of buffer A and 5 column volumes of 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8 (Buffer B). The column was then washed with 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 6.3 (Buffer C). The recombinant protein was eluted with 10-20 mL of 8 M urea, 0.1 M sodium-phosphate, 0.01 M Tris/HCl, pH 5.9 (Buffer D), followed by 10-20 mL 8 M urea, 0.1 M Sodium-phosphate, 0.01 M Tris/HCl, pH 4.5 (Buffer E). Fractions, 2 mL, were collected and analyzed on SDS-PAGE. The fractions which had cystatin band were combined (Anonymous, 1992)

Renaturation of recombinant fish cystatin

The combined fractions were dialyzed against 1 M urea in 0.05 M Tris/HCl, 0.005% Tween R-80, 1 mM reduced glutathione, 0.015 mM oxidized glutathione, pH 8.0 at 4 ^{0}C for 30 hr and an additional 18 hr in the same buffer, at half strength, for renaturation (Anonymous, 1992).

Purification of renatured fish cystatin under native condition

The renatured sample was loaded at 0.3-0.4 mL/min into another Ni-NTA resin which was previously equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. The column was washed with the same buffer until the A_{280} of the eluant was less than 0.01. The resin was also washed with 50 mM sodium phosphate, pH 6.0, containing 100 mM NaCl, 10% glycerol, until the A_{280} of the eluant was less than 0.01. The recombinant proteins were eluted with 50 mM sodium phosphate, pH 6.0, containing 100

mM NaCl, 10% glycerol, and 250 mM imidazole and 1 mL fractions were collected (Anonymous, 1992). The fractions containing active cystatin were determined by the modified method of Izquierdo-Pulido et al. (1994).

Fish cystatin inhibitory activity assay using papain-BANA system

The inhibitory activity of fish cystatin was determined against 0.45 μg of papain by using BANA as a substrate. Papain stock solution (3 mg/mL) was prepared and diluted with 4 % Brij (w/v) to 0.045 mg/mL concentration. Papain, 10 μL, and inhibitor, 30 μL, were mixed, and the total volume was brought to 400 μL with 100 mM phosphate buffer, pH 6, containing 1.33 mM EDTA and 2.7 mM cysteine. This mixture was preincubated at 40 ^{0}C for 5 min and 10 mL of 40 mg/mL BANA in DMSO was added to start the reaction. After 10 min incubation, the reaction was stopped by adding 400 μL of a mixture of 0.1 M Fast Garnet GBC base, 0.2 mM sodium nitrite, 2% Brij and 5 mM mersalyl acid. After incubating 10 min for color development, the reaction mixture was centrifuged at 8,000*xg* for 3 min. The red color produced by β-naphtylamide was measured at 520 nm as papain activity. The inhibitory unit was defined as liberation of 1 μmol β-naphtylamide per minute by papain (Izquierdo-Pulido et al., 1994).

Purification of cathepsin L from Pacific whiting muscle

Cathepsin-L was purified from parasitized Pacific whiting muscle according to the method of Seymour et al. (1994). To obtain sarcoplasmic fluid, 400 g finely chopped fish fillet was centrifuged at 5,000*xg* for 30 min. The supernatant was combined with an equal volume of McIlvaine's buffer (0.2 M sodium phosphate and 0.1 M sodium citrate), pH

5.5 and the mixture was heat-treated at 60 ^{0}C for 3 min in 100 mL fractions. The heat-treated mixture was centrifuged at 7,000*xg* for 15 min and the supernatant was dialyzed overnight against 20 mM Tris buffer, pH 7.5. After dialysis, saturated ammonium sulfate was added to adjust the concentration to 1 M. The sample was loaded onto the butyl-Sepharose column which was previously equilibrated with 20 mM Tris, pH 7.5, containing 1 M ammonium sulfate. After loading the sample, the column was washed with equilibration buffer until A_{280} was less than 0.05. The sample was eluted with 20 mM Tris, pH 5.5. Fractions, 5 mL, were pooled on the basis of activity and protein content (Seymour et al, 1994).

Proteolytic activity assay

The active fractions eluted from butyl-Sepharose column were assayed for proteolytic activity by the TCA-azo method (An et al., 1994a). Proteolytic activity was assayed using 2 mg azocasein as a substrate in 0.625 mL McIlvaine buffer, pH 5.5 and 25 μL of the enzyme. The final volume was adjusted to 1.25 mL with water. The reaction was carried out at 55 ^{0}C for 10 min and terminated by adding 0.2 mL cold 50% (w/v) TCA. After adding TCA to precipitate the unhydrolyzed protein, the reaction mixture was incubated for 15 min at 4 ^{0}C and centrifuged at 5,700*xg* for 10 min. The intensity of color of azo dye released was enhanced by adding 60 μL of 10 N NaOH and the absorbance of the solution was measured at 428 nm.

The activity on casein was determined by the procedure described for TCA-azo method but substituting casein for azocasein. After the reaction for 10 min at 55 ^{0}C, the

reaction was terminated by adding 50% (w/v) TCA. The TCA-soluble proteins were obtained by centrifugation and oligopeptide content in the supernatant was determined by the Lowry assay at 750 nm. The activity was expressed as nmoles of tyrosine released per min.

Inhibition assay against papain

Inhibitory activity of purified cystatins was measured, with slight modification, by the method of Abe et al. (1994). The assay buffer was 0.25 M sodium-phosphate, pH 6, containing 2.5 mM EDTA. Papain solution, 20 μg/mL, was activated with 25 mM sodium phosphate, pH 7 containing 20 mM β-mercaptoethanol at 40 ^{0}C for 10 min. The assay buffer, 0.2 mL, was mixed with 0.1 mL of the activated papain. After preincubation of the mixture with 0.2 mL of inhibitor at 40 ^{0}C for 5 min, the reaction was started by adding 0.2 mL of BANA and incubated at 40 ^{0}C for 10 min. The reaction was stopped by adding 1 mL of 2% (v/v) HCl in ethanol and the color was developed by adding 1 mL of 0.06% (w/v) *p*-dimethylaminocinnamaldehyde in ethanol. Reaction products were measured at 540 nm. The blank solution was prepared by substituting cystatin with water. The inhibitory activity was defined as a decreased amount of BANA-hydrolyzing activity per ml of inhibitor solution per hour. E-64, 0.19 μM was used as the positive control. For the negative control 5 μg casein and 5 μg BSA were used. The inhibitory activity was measured as the decrease in BANA-hydrolyzing activity. One "unit" of inhibitory activity was defined as the changes in absorbance of 1.0 at 540 nm per hour.

Active site titration

E-64 stock solution (1 mM) was prepared in DMSO and diluted to a working concentration (1 μM) with water. The assay buffer (0.25 M sodium-phosphate, pH 6, containing 2.5 mM EDTA) was mixed with 0.1 mL papain solution in 25 mM sodium phosphate, pH 7 containing 20 mM β-ME. After preincubation of the mixture with 0.2 mL of inhibitor at 40 ^{0}C for 5 min, the inhibitory activity was measured against papain as described above.

Gel electrophoresis

SDS-PAGE gels, 15 %, were performed according to Laemmli (1970) and 16.5% tricine SDS-PAGE gel was performed according to Schagger and Jagow (1987). Since soyacytatin has a low molecular weight, Laemmli's SDS-PAGE system did not give good resolution, therefore tricine SDS-PAGE was used. The composition and concentration of all buffers are given Table 1 and Table 2. The samples were boiled for 5 min in the SDS-PAGE treatment buffer (1:1, v/v) and applied on 15% and 16.5% polyacrylamide gels. The gels were run under a constant volt 150 V, on ice, using Bio-Rad Mini-Protean II unit (Bio-Rad, Hercules, CA).

Table 1. The composition of buffers and amounts used for tricine SDS-PAGE

Buffer and acrylamide-bisacrylamide mixture	**Composition**	**pH**
Buffer A (49 %T, 3 % C)	48% (w/v) acrylamide 1.5% (w/v) bisacrylamaide	
Buffer B (gel Buffer)	3 M Tris 0.3 % SDS	pH=8.45
Lower tank buffer	1 M Tris (5x)	pH=8.9
Upper tank buffer	0.1 M Tris 0.1 M Tricine 0.1 % SDS	pH=8.25

Table 2. The composition 16.5% gel

Material	**Running gel**	**Separating gel**
Buffer A	3 mL	1 mL
Buffer B	3 mL	3.1 mL
10 % SDS	90 µL	125 µL
10 % Ammonium per sulfate	35 µL	100 µL
Water	2.9 mL	8 mL
TEMED	5 µL	5 µL
Total	9 mL	12.5 mL

Temperature stability

Both purified egg white cystatin and soyacystatin were incubated at 0, 20, 30, 40 and 60 ^{0}C, respectively, for 30 min and cooled in ice prior to inhibitory activity assay using papain BANA system.

pH stability

Both purified egg white cystatin and soyacystatin were incubated with McIlvaine's buffer in the pH range of 4-10 at room temperature for 15 min prior to inhibitory activity assay using papain BANA system.

The effect of pH on the inhibitory activity

To 0.2 mL of 0.25 M sodium phosphate in the pH range 4-9, containing 2.5 mM EDTA was added 0.1 mL papain solution in 25 mM sodium phosphate, pH 7 containing 20 mM β-mercaptoethanol. After preincubation of the mixture with 0.2 mL of inhibitor solution at 40 ^{0}C for 5 min, the residual activity of papain was measured, as described above, using BANA as a substrate.

Isoelectric focusing

Isoelectric focusing was performed in premade agarose gels (FMC Corp., Rockland, ME). The wicks were soaked in 1 M NaOH, as catholyte, and 0.5 M acetic acid, as anolyte. Samples were applied on the gel with the sample applicator mask placed midway between the anode and cathode. Papain solution was prepared as 80 μM in 2 mM dithiothreitol and 3 μL from that solution was loaded onto then gel. Egg white, 1.04 μg,

was used and soyacystatin, 1.33 μg, was used. After the gel was prefocused with 1 W constant power for 10 min, it was focused for 40 min at 25 W constant power with 1000 V limit using a Thin-Layer Isoelectric Focusing (Desaga Heidelberg). After focusing, the gel was fixed for 1 hr with 36% (v/v) methanol, 6% (w/v) trichloroacetic acid and 3.6% (w/v) sulfosalicylic acid. The fixed gel was washed with distilled water for 15 min to remove residual fixative and ampholytes. The gel was dried at 55 ^{0}C for 1.5 hr. Protein bands were stained with 0.1% Coomasine brillant blue R-250 for 30 min and destained in 25% (v/v) of ethanol and 9% (v/v) of acetic acid for 3 min. The destained gel was dried at 55 ^{0}C for 30 min. To estimate the isoelectric point of the proteins a broad range pI standards were used.

Inhibition activity assay for cathepsin L

Cathepsin L assay was performed according to Barrett and Kirschke (1981), with slight modification. Z-Phe-Arg-Nmec was used as a specific substrate for cathepsin L. Cathesin L was diluted with 0.1% (w/v) Brij to final volume of 500 μL and mixed with 250 μL of the assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA and 8 mM dithioerythritol, pH 5.5). This mixture was preincubated at 30 ^{0}C for 1 min. After preincubation, 250 μL of 20 μM of the substrate was added to start the reaction. After reacting for precisely 10 min at 30 ^{0}C, 1 mL of 5 mM iodoacetic acid was added to the reaction mixture to stop the reaction. The flourescence of the free aminomethylcoumarin was determined by emission at 460 nm and exitation at 370 nm using Perkin Elmer Lininescence Spectrometer Model (Norwalk, CT)

Active site titration

The absolute cathepsin L concentration was determined by active site titration using the method of Barrett and Kirschke (1981), with slight modification. Enzyme solution, 25 μL, and 25 μL of E-64 solution was mixed with 50 μL buffer (0.2 M sodium phopshate, 0.1 M citrate, 4 mM disodium EDTA and 8 mM dithioerythritol, pH 7). The mixture was preincubated at 30 ^{0}C for 5 min. After incubating at 30 ^{0}C for 5 min, 500 μL of 0.1% Brij solution and 200 μL of the assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA and 8 mM dithioerythritol, pH 5.5) were added. The reaction mixture was reeqilibrated to 30 ^{0}C for 1 min and the residual activity of cathepsin L was measured as described above.

Inhibition assay against cathepsin L

Inhibition of cathepsin L by cystatin was tested according to Barrett and Kirschke (1981), with slight modification. Cathepsin L, 400 ng determined by active site titration, was diluted with 0.1% Brij to total volume 25 μL. Enzyme solution, 25 μL, was mixed with 25 μL of a suitable dilution of the inhibitor solution. To the enzyme-inhibitor solution, 50 μL buffer (0.2 M sodium phosphate, 0.1 M citrate, 4 mM disodium EDTA and 8 mM dithioerythritol, pH 7) was added. The purified soyacystatin, purified egg white cystatin and soyacystatin cell extracts were used as inhibitors. Casein was used as a negative control, while E-64 was used as a positive control. After incubation at 30 ^{0}C for 5 min, 500 μL of 0.1% Brij solution and 200 μL of the assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA and 8 mM dithioerythritol, pH 5.5)

were added. The reaction mixture was reequilibrated to 30 ^{0}C for 1 min and the residual activity of cathepsin L was measured as described above.

Autolysis assay

Soyacystatin, expressed in *E. coli.*, was prepared as described above and cells were harvested by centrifugation. Harvested cells were sonicated using 10 mL of 10 times diluted McIlvaine's buffer, pH 7, in ice until a clear solution was obtained. Sonicated cell extract was centrifuged at 7,000*xg* for 10 min and the resulting supernatant was stored at -80 ^{0}C with glycerol.

Autolysis assay was carried out according to Morrissey et al. (1993). Finely chopped Pacific whiting (3 g) and Pacific whiting surimi (3 g) were mixed with 3 mL inhibitor solutions and incubated at 55 ^{0}C for 30 and 60 min for Pacific whiting fish fillet and Pacific whiting surimi, respectively. The autolytic reaction was stopped by adding 5% (w/v) cold TCA solution. To precipitate unhydrolyzed proteins, the solution mixture was incubated at 4 ^{0}C for 15 min and centrifuged at 6,100*xg* for 15 min. The supernatant, which had TCA-soluble proteins was analyzed for oligopeptide contents with Lowry assay (Lowry et al., 1951), and the activity was expressed as nmoles tyrosine released per minute. Sample blanks containing all components were kept on ice and used to correct for oligopeptide content originating from Pacific whiting, Pacific whiting surimi and proteinase inhibitors.

For positive control 0.1mM E-64, BBP, and egg white were used. Casein and water were run, as negative control. All treatments were run duplicate.

$$\% \text{ Inhibition} = \frac{(C_{55}-C_0) - (I_{55}-I_0)}{(C_{55}-C_0)} \times 100$$

C_{55} = nmoles tyrosine/min surimi without inhibitor at 55 ^{0}C

C_0 = nmoles tyrosine/min surimi without inhibitor at 0 ^{0}C

I_{55} = nmoles tyrosine/min surimi with inhibitor at 55 ^{0}C

I_0 = nmoles tyrosine/min surimi with inhibitor at 0 ^{0}C

Protein content

Protein content was determined according to Lowry et al (1951) using bovine serum albumin as a standard. The protein content was determined on the basis of A_{280} value according to the following equation

Protein content (mg/mL) = A_{280} /1.4

RESULTS AND DISCUSSION

Purification of Recombinant Soyacystatin

The cell (pETNM$^{8\text{-}103}$) was tested for the expression of recombinant soyacystatin on SDS-tricine PAGE. As seen in Figure 1, the soyacystatin was visible after induction with IPTG. The result showed that the cell had a high amount of recombinant cystatin. After induction with IPTG, recombinant soyacystatins were purified from the cells using hydrophobic chromatography on phenyl-Sepharose, as the first step and ion exchange chromatography on DEAE, as the second step. In ion exchange chromatography, charged molecules bind, reversibly, onto an ion exchange resin. Then they are eluted by increasing the salt concentration which disrupts the electrostatic interaction between the ion exchange resin and the molecules. Recombinant soyacystatin started to elute from DEAE at 0.146 M NaCl (Figure 2). The recombinant soyacystatin was purified 4.33 fold with 39.7% yield (Table 3). From 500 mL cell culture, 19.95 mg soyacystatin in 30 mL was obtained with 39.7% yield (Table 3). On tricine SDS-PAGE, the purified soyacystatin showed as a strong visible band (Figure 3). Purified cystatin was tested against papain. On the basis of 1:1 stoichiometric reaction, purified cystatin was found to be 99% active.

Three cysteine proteinase inhibitor cDNA clones, i.e., pL1, pR1 and pN2, have been isolated from soybean embryo library. It was reported that the amino acid sequences of pR1 was 67% identical with pL1; pL1 was 61% identical with pN2; and pR1 was 64 identical with pN2 (Botella et al., 1996). pR1 and pN2 had more inhibitory

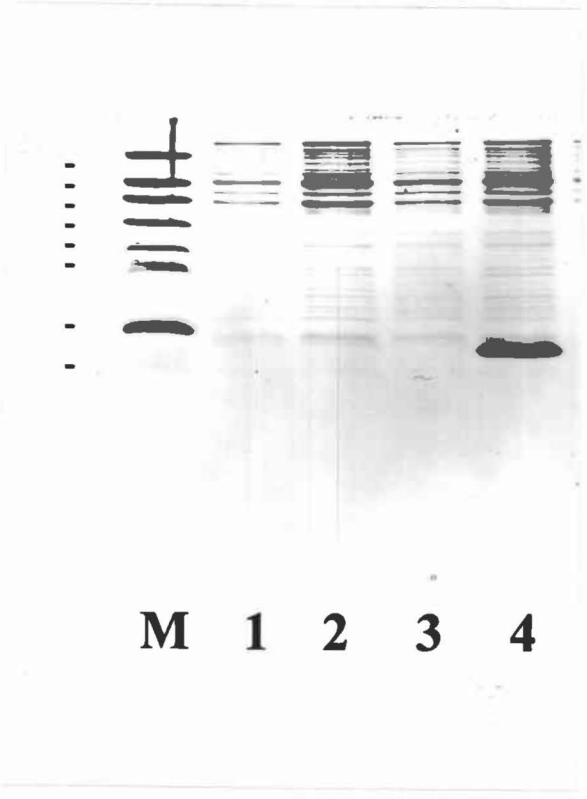

Figure 1. Expression of soyacystatin on the tricine SDS-PAGE. Samples, 5 μL, were taken from before and after induction, boiled with 5 μL SDS treatment buffer and loaded on the gel. (M) Low molecular weight marker; (1) uninduced cells containing plasmid without cystatin; (2) induced cells with plasmid without cystatin; (3) uninduced cells with recombinant soyacystatin; (4) induced cells with recombinant soyacystatin

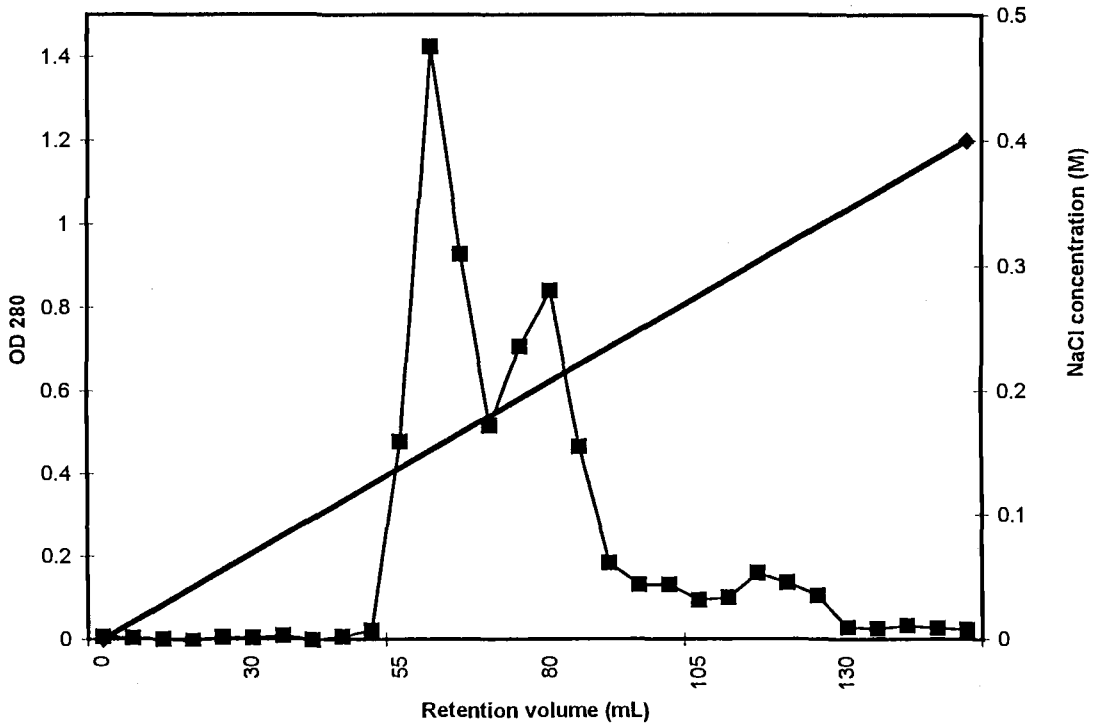

Figure 2. Elution profile of recombinant soyacystatin from DEAE. Recombinant soyacystatin was eluted from DEAE column with linear gradient of 0-0.4 M NaCl in 10 mM Tris, pH 8.8

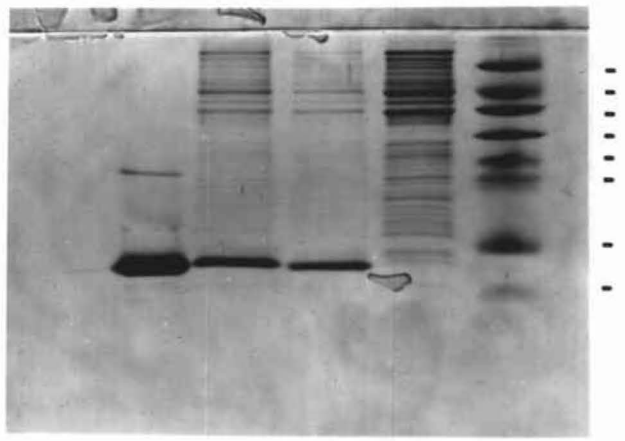

Figure 3. Various stage of purification of recombinant soyacystatin on SDS-tricine PAGE. (1) 5 μg purified recombinant soyacystatin; (2) 5 μg ammonium sulfate precipitated soyacystatin cell extract; (3) 5 μg induced recombinant soyacystatin cell extract; (4) 5 μl of uninduced recombinant soyacystatin cell extract; (M) low molecular weight marker.

Table 3. Purification table of soyacystatin. The inhibitory activity was measured as decrease in BANA hydrolyzing activity. One unit of inhibitory activity was defined as the changes in absorbance of 1.0 at 540 nm per hour.

Purification Steps	Volume (mL)	Activity (U/mL)	Total Activity (U)	Prot. Conc. (mg/mL)	Total Protein (mg)	Spec. Act. (U/mg)	Purification Fold	Yield (%)
Sonicated cell	17	44307	753219	12.5	212.5	3544.5	1.00	100
Ammonium sulfate precipitation	20	34180	693600	4.50	90.00	7706.6	2.17	92.0
DEAE	30	9971.0	299130	0.665	19.95	15341	4.33	39.7

activity than pL1 against gut protease of the third-instar larvae of western corn rootworm (Zhao et al., 1996). In this study, pN2 showing higher inhibitory activity was used. Since the soyacystatin cDNA clone did not encode a methionine for the initiation of translation (Botella et al., 1996), the insert was modified to produce peptide by Koiwa et al. (1998).

Purification of Egg White Cystatin

Egg white cystatin was purified by affinity chromatography. Affinity chromatography is a powerful tool in isolation and purification of enzymes, antibodies, antigens, nucleic acid, polysaccharides, coenzyme, vitamin-binding proteins, repressor proteins, transport proteins, drug and other biochemical substances (Robyt and White, 1990). For this study, cm-papain-Sepharose, which is capable of isolating cystatin from numerous egg white proteins, was used as the affinity media. For the purification, the egg white was treated with NaCl, and pH was adjusted to 6.5 to remove ovomucin. After the salt treatment, egg white was stirred with cm-papain-Sepharose overnight, which allowed papain to bind covalently to egg white cystatin. By taking advantage of the instability of the lysosomal cysteine proteinase in alkaline conditions, the bound cystatin was eluted from cm-papain-Sepharose by increasing pH to 11.5.

Egg white cystatin was purified from 12 pooled egg whites with 5.90% yield and 343 purification fold (Table 4 and Figure 4). The purification step is summarized in Table 4 and Figure 4. The yield was low and compared with the previously reported work of Anastasi et al. (1983). The investigators purified the egg white cystatin with 34.9 % yield.

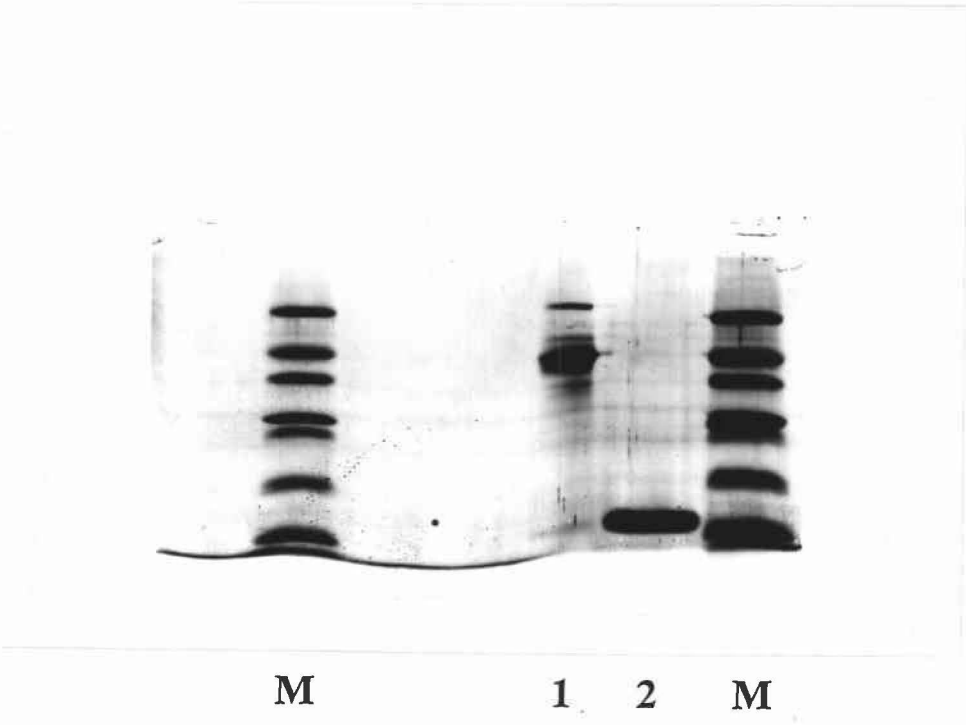

Figure 4. Egg white purification step on the SDS-PAGE. (M) Low molecular weight marker; (1) egg white proteins; (2) purified egg white cystatin; (M) low molecular weight marker

Table 4. Purification table for egg white cystatin. The inhibitory activity was measured as a change in reaction rate. One unit was defined change in absorbance of 1.0 per minute

Purification Steps	Volume (mL)	Activity (U/mL)	Total activity (U)	Prot. conc. (mg/mL)	Total protein (mg)	Spec. Act. (U/mg)	Purification fold	Yield (%)
Egg White	300	0.707	212	99.0	29700	0.00714	1.00	100
Cm-papain Sepharose	9.80	1.27	12.5	0.520	5.10	2.45	343	5.90

The low yield encountered in this study was speculated to be caused by the oversaturation of the resin with cystatin.

Egg white cystatin was composed of two forms. The two forms were not distiquishable from each other on SDS-PAGE (Figure 4). However, they are easily separated on an isoelectric focusing gel (Figure 18). Cystatin form 1 and form 2 have isoelectric points of 6.6 and 5.84, respectively. These values are very similar to those reported by Anastasi et al (1983). They reported that cystatin form 1 and 2 had isoelectric points, 6.5 and 5.6, respectively.

Purification of Fish Cystatin

Rainbow trout cystatin was expressed as a fusion protein with a 6xHis tag on the N-terminus (Li, 1998). With the presence of His tag, the recombinant proteins were easily purified by using affinity chromatography. Ni-NTA resin has a high affinity for proteins and peptides which contain 6xHis affinity tag. Recombinant fish cystatin was purified under denaturing conditions for higher yield. Sometimes proteins can become insoluble and located in inclusion bodies of bacterial cells. Thus, before purification, solubilization of protein by denaturing agents is generally recommended for higher recovery (Anonymous, 1992). The recombinant protein was first treated with a high concentration of denaturing agents, i.e., GuHCl and urea, and was purified under denaturing condition. After purification of the denatured protein, the protein was renatured in a low concentration of denaturing agents to recover the activity by promoting proper folding of disulfide bond. The renaturation process took 30 hr in 1 M urea, 0.05 M Tris/HCl,

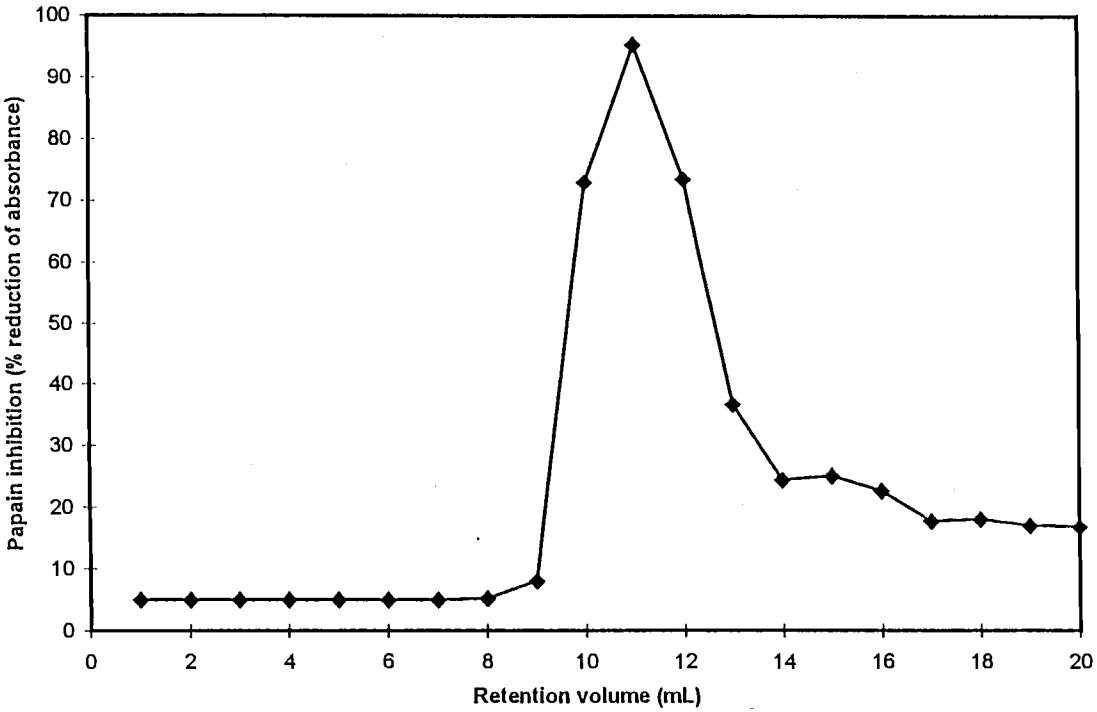

Figure 5. Elution profile of recombinant fish cystatin from Ni-NTA resin with 50 mM sodium phosphate, pH 6 containing 100 mM NaCl, 10% (v/v) glycerol and 250 mM imidazole. Each fraction was tested against 0.45 μg papain using BANA as a substrate and monitored for changes in absorbance at 520 nm. The percent reduction of absorbance at 520 nm was calculated as the inhibition of papain and plotted against retention volume.

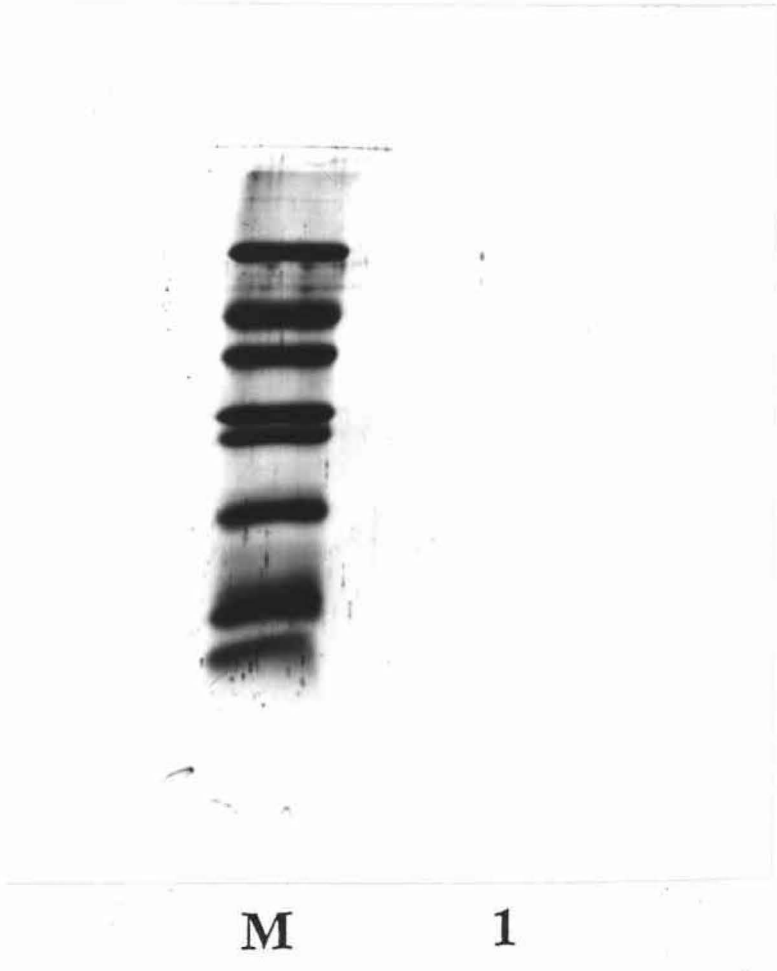

Figure 6. Detection of recombinant fish cystatin on SDS-PAGE. (M) Low molecular weight marker and (1) 1 μg of recombinant fish cystatin.

0.005% Tween R-80, 1 mM reduced glutathione and 0.015 mM oxidized glutathione, pH 8 and additionally for 18 hr in the same buffer at half strength. After refolding, the recombinant protein underwent another affinity chromatography step under native condition to obtain higher purity. At the end of the step, the activity of fraction was tested against 0.45 μg papain, and active fractions were pooled together (Figure 5). Although only two purification steps were needed, the long refolding process was required with low recovery. Only 0.21 mg of purified cystatin was obtained from a 500 mL cell culture. The protein level was quite low, 0.071 mg/mL, as estimated by A_{280}. The SDS-PAGE result also showed that the purified sample was very dilute, as determined by the weak band observed on the gel (Figure 6).

Purification of Cathepsin L

Two forms (P-I and P-II) of cathepsin L were purified from Pacific whiting muscle with 13.7 and 25.7 fold, respectively. The purification steps are summarized in Table 5 and Figure 7. The heat treatment step served to inactivate and remove heat-labile proteolytic enzymes, myofibrillar and sarcoplasmic proteins. Hydrophobic chromatography on butyl-Sepharose was effective in separating the activity of two peaks designated as P-I and P-II (Seymour et al., 1994). Fractions from both peaks containing active enzymes, as analyzed by TCA-Azo assay were pooled and applied on SDS-PAGE. As seen on SDS-PAGE gel, P-I fraction contained the low molecular weight components while P-II had only one band whose molecular weight was 28,800. An et al. (1995) reported that P-I was complex-formed with an inhibitor and PII was a free enzyme. An et

Table 5. Purification table of Pacific whiting protease. The activity was defined as 1 nmol of Tyrosine equivalent solubilized/min.

Purification Steps	Volume (mL)	Activity (U/mL)	Total Activity (U)	Prot. Conc. (mg/mL)	Total Protein (mg)	Spec. Act. (U/mg)	Purification Fold	Yield (%)
Fish Juice	125	3012.5	376562.5	29.39	3673.75	102.5	1.00	100
Heat Treatment	248	2321.0	575608.0	14.81	3672.88	156.7	1.52	152
Dialysis	200	2175.0	435000.0	6.310	1262.00	344.7	3.36	115
Ammonium Sulfate treatment	160	1692.0	270720.0	4.110	657.60	411.6	4.01	72.0
Butyl-Sepharose								
PI	18.0	1925.0	34650.00	1.370	24.660	1405	13.7	9.20
PII	74.0	1056.0	78144.00	0.400	29.600	2640	25.7	21.0

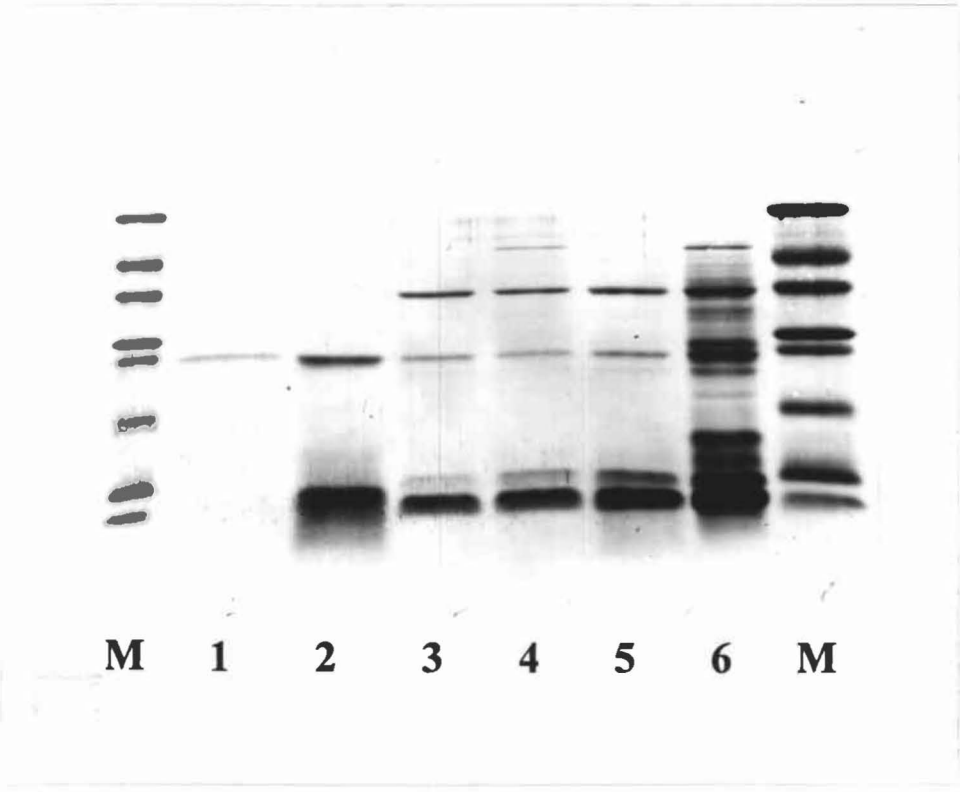

Figure 7. Various purification stage of Cathepsin L analyzed on SDS-PAGE. (M) Low molecular weight marker; (1) 4 μg of PII; (2) 6.85 μg of PI; (3) 4.31 μg of protein after ammonium sulfate treatment; (4) 3.155 g of protein after dialysis; (5) 7.4 μg of fish juice after heat treatment and (6) 7.34 μg of fish juice

al. (1995) showed that low pH treatment, pH 3.3, increased the activity of P-I by dissociating the proteinase inhibitor complex, however it had no effect on P-II. This result indicates that P-II is a pure enzyme. We purified the P-I and P-II with 9.2 and 21% yield, respectively. Purification fold is 13.7 for P-I, and 25.7 for PII (Table 5). Those results are very close to the work previously reported by Seymour et al. (1994) who purified PI and PII with 24.0 and 26.0 yield and 16.2 and 20.4 purification fold, respectively.

Protein Content

Protein content of the purified recombinant cystatins were determined by the method of Lowry et al (1951). However due to the low recovery, the protein content of recombinant fish cystatin was estimated based on the A_{280} value.

Table 6. The protein content of purified cystatins

Samples	Protein concentration (mg/mL)
Recombinant soyacystatin	0.665
Soyacystatin cell extract	12.5
Egg white cystatin	0.520
Recombinant fish cystatin	0.071

Inhibitory Activity

Inhibitory activity of cystatins was determined according to Abe et al (1994) by using BANA as a substrate against 2 μg of papain per assay. The amount of purified soyacystatin (Figure 8) and egg white cystatin (Figure 9) required to obtain 50% inhibition of papain was 0.245 μg and 0.455 μg, respectively. With soyacystatin crude cell extract (Figure 10), 0.81 μg was needed for achieving 50% inhibition of papain.

To compare the inhibitory activity of recombinant rainbow trout fish cystatin with those of soyacystatin and egg white cystatin, fish cystatin was tested against 2 μg papain. We found that 3.57 μg (50 μL) inhibited 90% of papain activity used in the assay (Figure 11). However, we could not continue to optimize the concentration of recombinant fish cystatin to obtain 50% inhibition because of its low yield and low activity. Therefore, we compared the amount of the other cystatins with fish cystatin to obtain 90% inhibition of papain activity. To achieve this level of papain inhibition, 3.57 μg of recombinant fish cystatin, 0.429 μg of recombinant soyacystatin and 0.810 μg of egg white cystatin were needed (Figure 11). As seen in Figure 11, the recombinant fish cystatin needed a higher amount to obtain the same level of inhibition (90%). The inhibitory activity of fish cystatin was about 8.2 times less than recombinant soyacystatin and 4.4 times less than egg white cystatin. The protein content of purified fish cystatin was also low. This results makes the fish cystatin unfeasible for further characterization and its study was discontinued.

On the contrary to fish cystatin, soyacystatin cell extract (sonicated cell) had a high level of activity without purification. To inhibit 90% activity of papain, 1.50 μg of

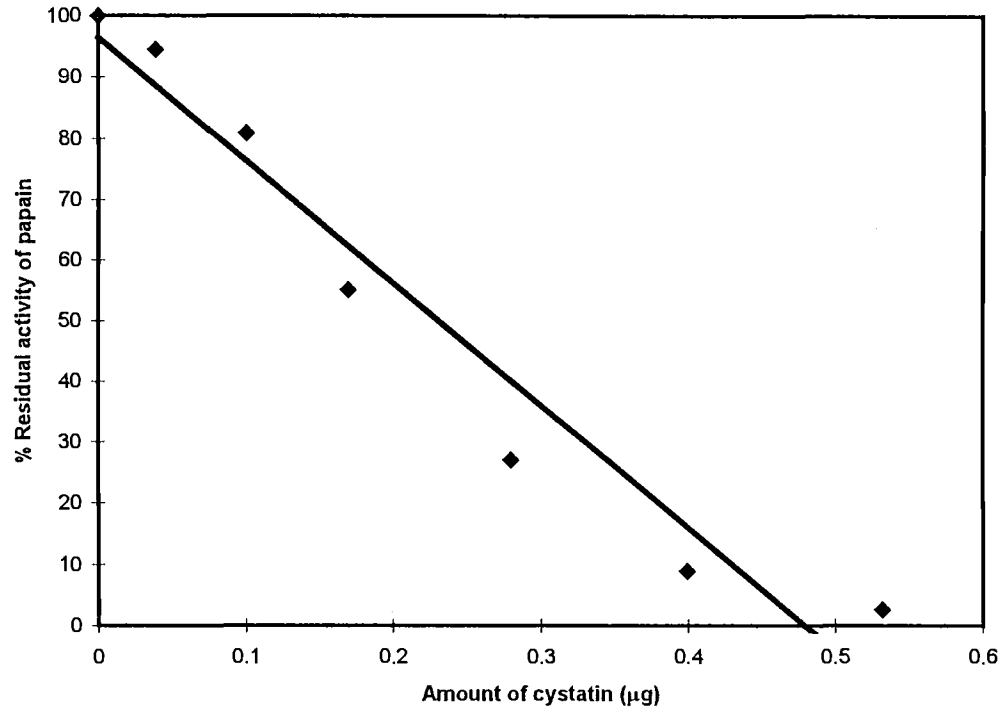

Figure 8. The inhibitory activity of soyacystatin against papain. Inhibitory activity of soyacystatin was tested against 2 µg papain using BANA as a substrate at 40 ^{0}C for 10 min.

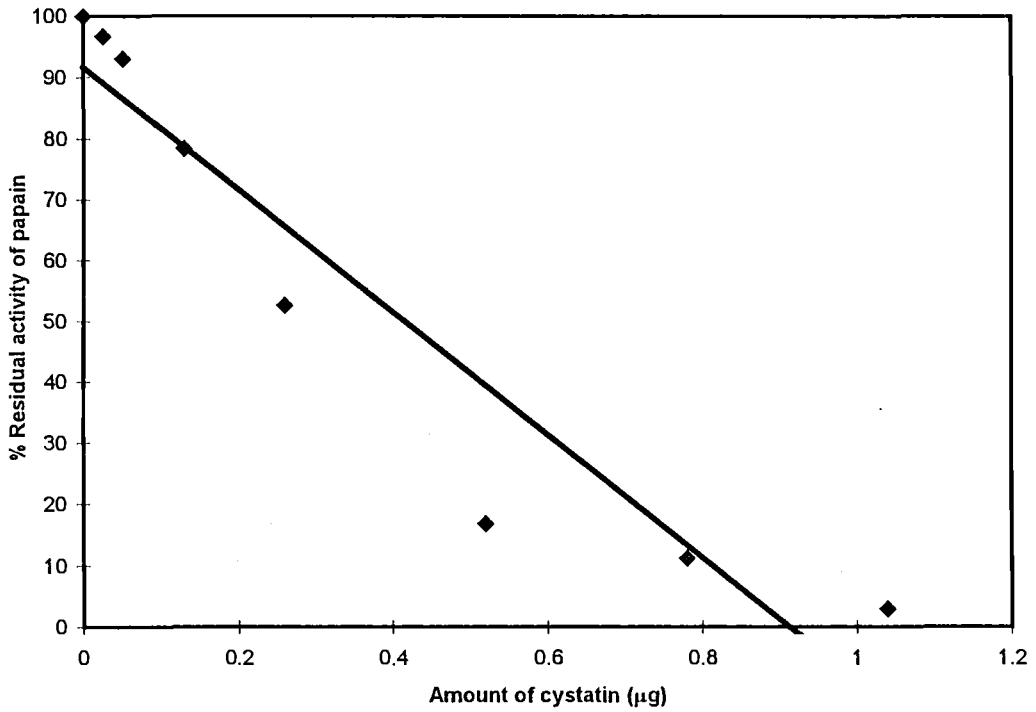

Figure 9. The inhibitory activity of egg white cystatin against papain. Inhibitory activity of egg white cystatin was tested against 2 μg papain using BANA as a substrate at 40 ^{0}C for 10 min

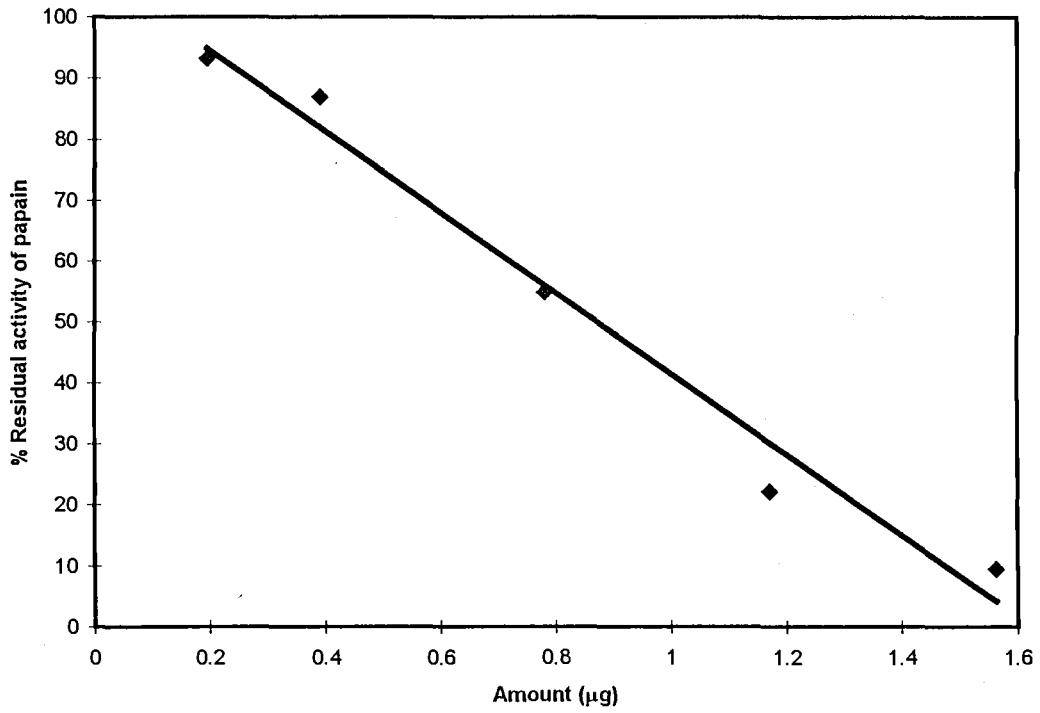

Figure 10. The inhibitory activity of soyacystatin cell extract against papain. Inhibitory activity of soyacystatin cell extract was tested against 2 µg papain using BANA as a substrate at 40 ^{0}C for 10 min

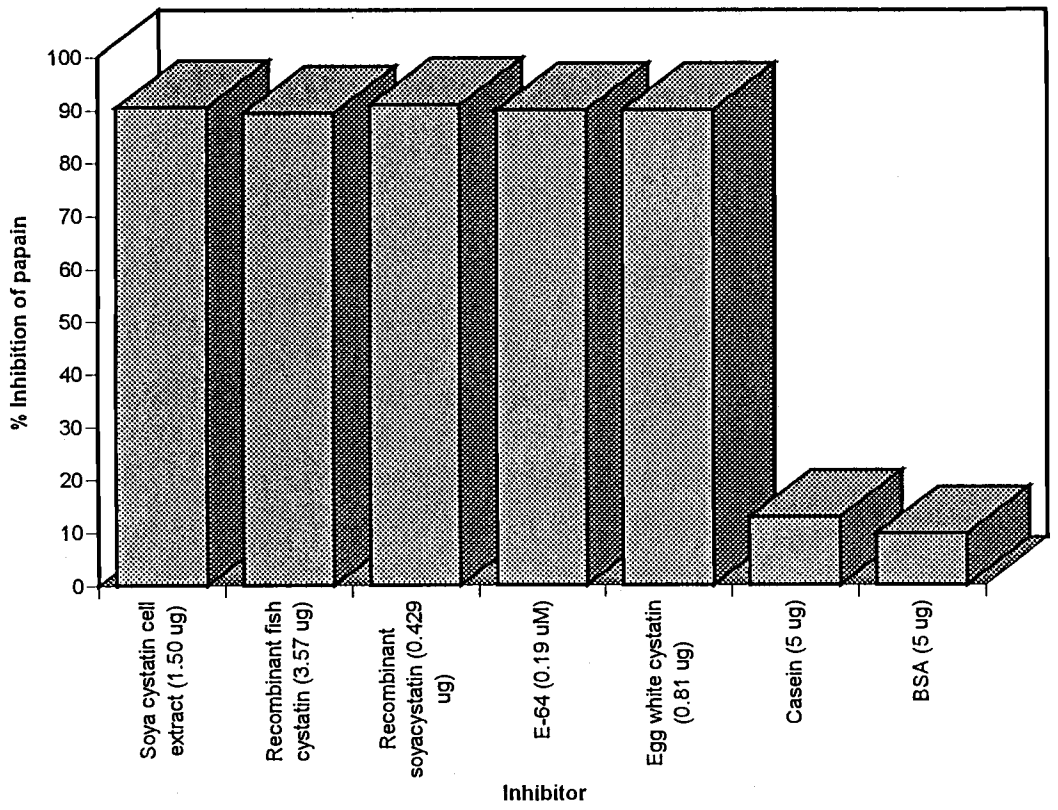

Figure 11. Comparison of inhibitory activities of cystatins against 2 μg papain at 40 ^{0}C for 10 min.

the cell extract was sufficient. The protein concentration was also high, 12.5 mg/mL. This property enabled us to use sonicated cell extract directly in testing with surimi. Casein and BSA were run as negative controls to determine if inhibition of papain resulted from an increased amount of protein content in the assay system. As seen in Figure 11, casein and BSA lowered proteolytic activity by nonspecific competitive inhibition; however, the activity was not as high as the specific inhibitors.

Active Site Titration

E-64 is an extremely effective cysteine proteinase inhibitor. Absolute molarity of cysteine proteinases can be determined by stoichiometric titration with E-64 (Barrett and Kirsche, 1981). E-64 titration with papain first allows the calculation of active concentration of papain used in the assay (Figure 12). Based on the absolute concentration of papain, the absolute activity of soyacystatin and egg white cystatin can be calculated, respectively (Table 7). Cystatin and papain form a complex at an equimolar ratio. We found that papain used in the assay was 50.0% active, the purified egg white cystatin was 65.5% active, and soyacystatin was 99% active.

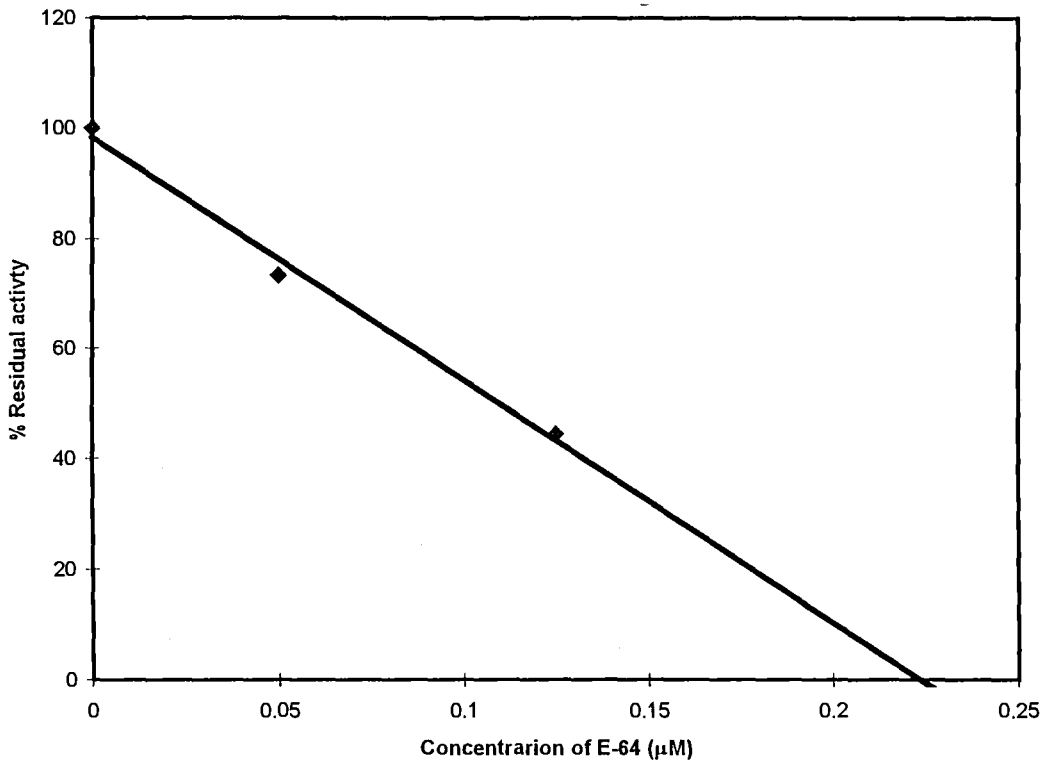

Figure 12. E-64 titration of papain. E-64 titration of papain was carried out using BANA against 2 μg papain at 40 ^{0}C for 10 min.

Table 7. The absolute concentration of papain and inhibitors

Name	Concentration used in the experiment (picomole)	Active portion Concentration (picomole)	% Active concentration
Papain	89.2	44.6	50.0
E-64	44.6	44.6	100
Egg white cystatin	68.0	44.6	64.0
Soya cystatin	45.0	44.6	99.0

Temperature Stability

Temperature stability of egg white and soyacystatin was determined (Figure 13). Egg white cystatin was stable up to 30 ^{0}C and lost 36% of its initial activity by heating at 30 ^{0}C for 30 min. At temperatures above 30 ^{0}C, it lost its activity faster. By incubating at 60 ^{0}C for 30 min, egg white cystatin lost 70 % of its activity

Soyacystatin lost its activity by heating faster than egg white cystatin (Figure 13). For example, soyacystatin lost 22.5% of its activity at 20^{0}C while egg white cystatin lost only 15% of its activity at this temperature. At 30 and 60 ^{0}C, soyacystatin lost 36 and 80% of its activity, respectively.

Egg white cystatin is reported to be heat stable (Fossum and Whitaker, 1968) and according to the above results, egg white cystatin is more stable than soyacystatin. Egg white cystatin has two disulfide bonds while soyacystatin has no disulfide. A disulfide bond is a covalent crosslink which increases protein stability. It controls and limits the

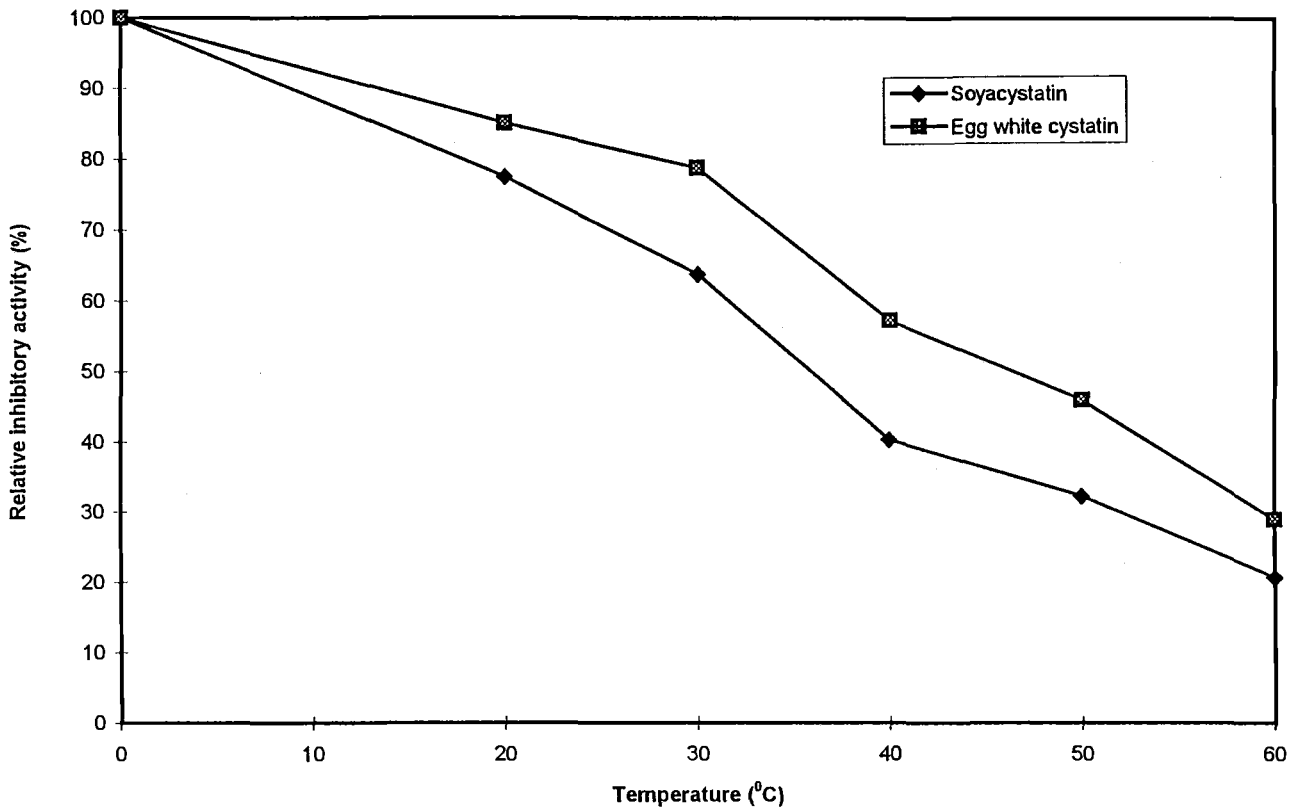

Figure 13. Thermal stability of soyacystatin and egg white cystatin. Egg white cystatin and soyacystatin were incubated at 0, 20, 30, 40, 50 and 60 °C for 30 min and immediately cooled in ice prior inhibitory activity assay.

unfolded stages of the protein structure by decreasing the conformational entrophy and increasing the free energy (Creighton, 1993). From Figure 13, it is shown that both cystatin forms can maintain their inhibitory activity after heating for 30 min at 60 ^{0}C, although their inhibitory activity was reduced. The most interesting characteristic in all four types of cystatin family is that they can survive in conditions which cause most proteins to be unstable (Barrett et al., 1986). These conditions are extreme pH and high temperature.

pH Stability

pH stability of both cystatins was tested by incubating them at different pH. Figure 14 shows the pH stability of egg white cystatin and soyacystatin. Both cystatins were relatively stable in the wide range of pH although they belong to different cystatin families. When the pH stability of egg white and soyacystatin was compared, soyacystatin seemed slightly more stable at a high pH. For example, soyacytatin lost 12 % of its activity, while egg white cystatin lost 20% at pH 9.

Brzin et al. (1983) isolated and characterized stefin from human polymorphonuclear granulocytes. They found that stefin was quite stable at alkaline pH even at elevated temperatures. Oryzacystatin was found to be stable at a broad pH range, although it was most stable between pH 4 and 8. At pH 10, oryzacystatin significantly lost its activity (Izquierdo-Pulido et al., 1994). Egg white cystatin is reported to be stable in alkaline conditions as a result, the elution of egg white cystatin from cm-papain-Sepharose column can take place at high pH, usually pH 11-12 (Anastasi et al., 1983; Barrett et al., 1986).

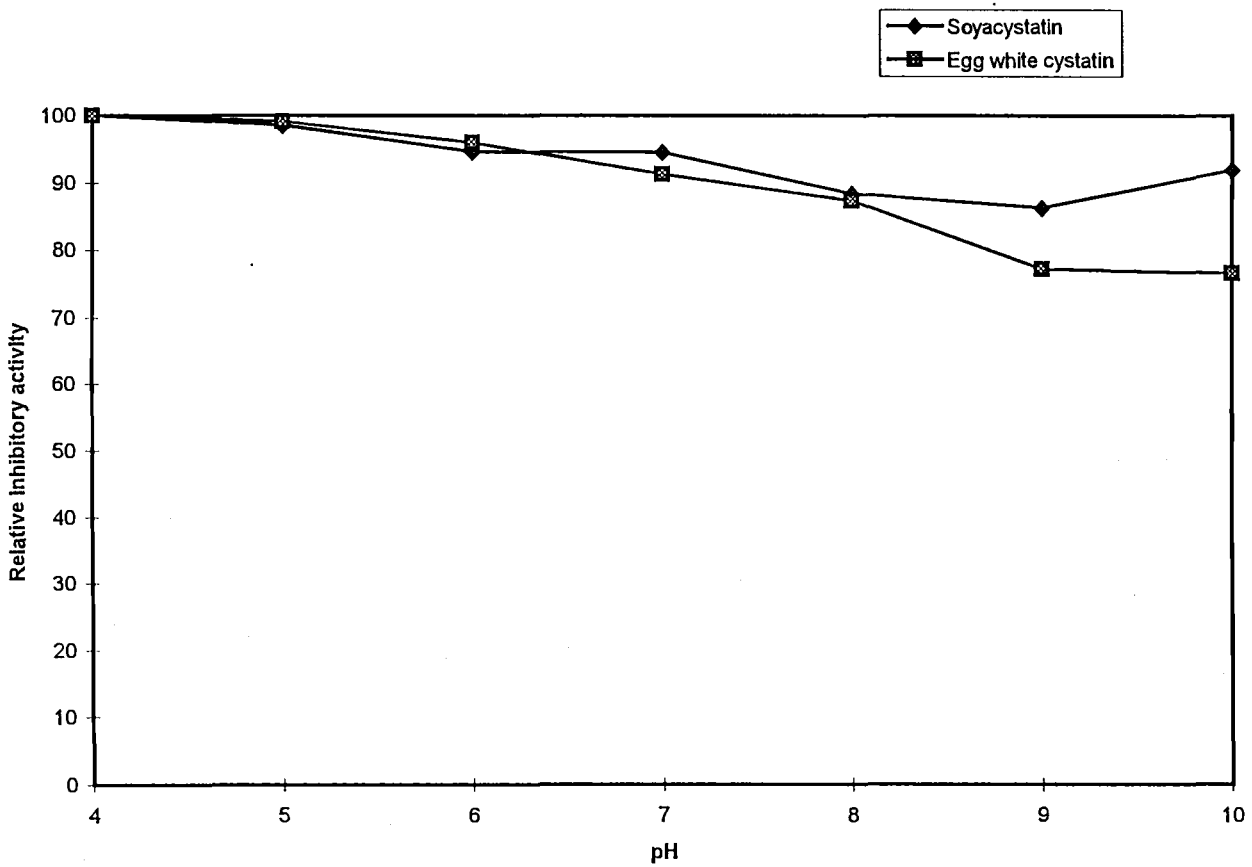

Figure 14. pH stability of soyacystatin and egg white cystatin. Egg white cystatin and soyacystatin were incubated in McIlvaine's buffer in the pH range of 4-10 at room temperature for 15 min prior to inhibitory activity assay.

Effect of pH on the Inhibitory Activity

Figure 15 shows the activity of papain alone and in the presence of soyacystatin and egg white cystatin, respectively at different pH. As seen from Figure 15, papain showed high activity between pH 4 and 8 as determined absorbance at 540 nm. However, above pH 9, it lost its activityat a faster rate. Since there was no meaningful papain activity below pH 4 and above pH 9, they have been excluded from the study. In the presence of soyacystatin or egg white cystatin, effective inhibition of papain is shown by both cystatin compounds between pH 4 to 9. Even at high pH, the inhibition of papain by cystatin is the same as in the neutral pH. As seen in Figure 16, the inhibition of papain increased with increases in pH. Since alkaline pH does not effect the activity of cystatins, the cystatins continued to inhibit papain at high pH.

Soyacystatin at pH 4 to 9 showed a high capability to inhibit papain (Figure 16). Although the percent inhibition is high at pH 6 and 7, soyacystatin can still inhibit the papain well at pH 4 and 5. On the contrary, egg white cystatin seems to be less efficient than soyacystatin for inhibiting papain at slightly acidic pH. At pH 4, soyacystatin inhibited 90% of papain activity while egg white cystatin inhibited only 70% activity.

The ability of soyacystatin to inhibit the proteinase at slightly acidic pH showed that soyacystatin could inhibit cathepsin L which has optimum pH at 5.5. Cathepsin L is the major enzyme which causes softening in the surimi gel.

Figure 16 showed that at neutral pH soyacystatin bound papain very well. Since surimi has a neutral pH and soyacystatin has a high inhibitory activity against papain at neutral pH, soyacystatin is giving promising result for inhibiting proteinase of surimi.

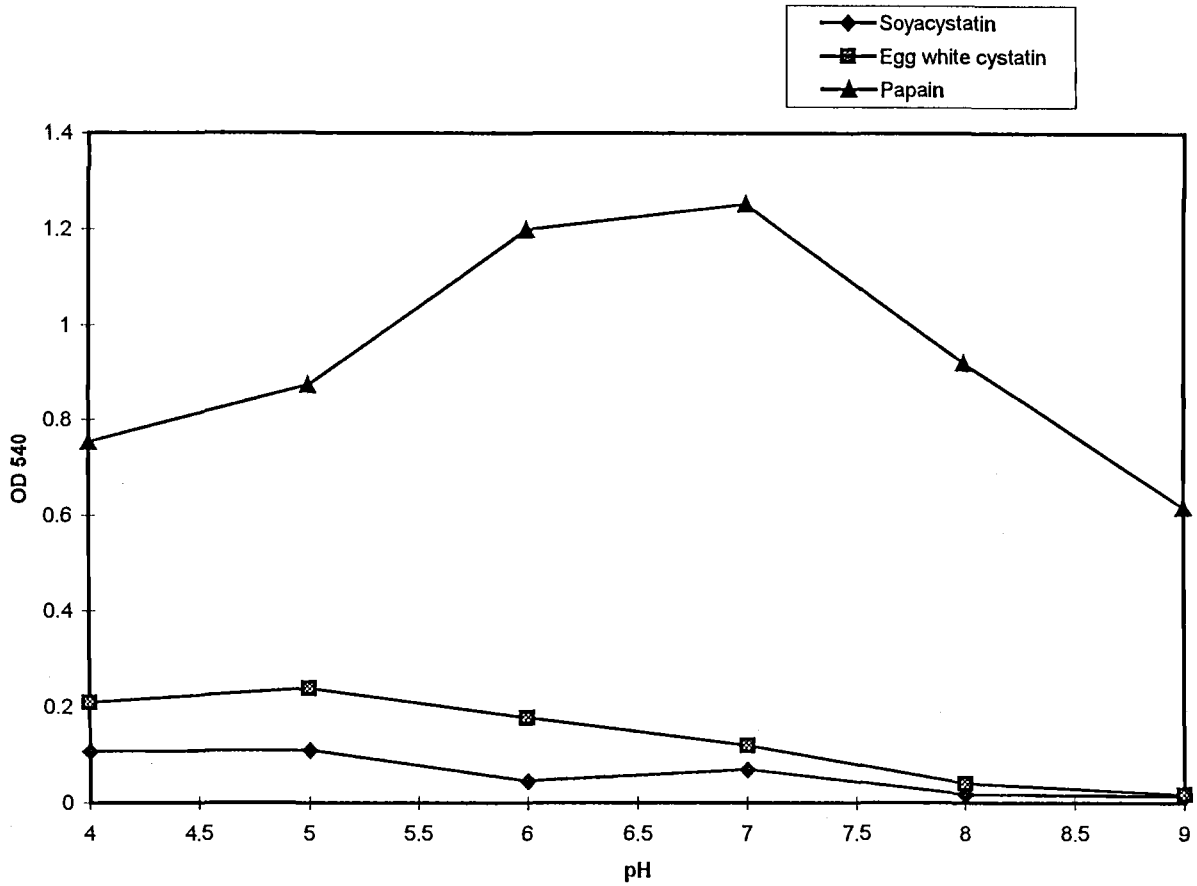

Figure 15. The activity of papain with and without cystatins at different pH. Papain and papain with cystatins were analyzed in pH range of 4 to 9

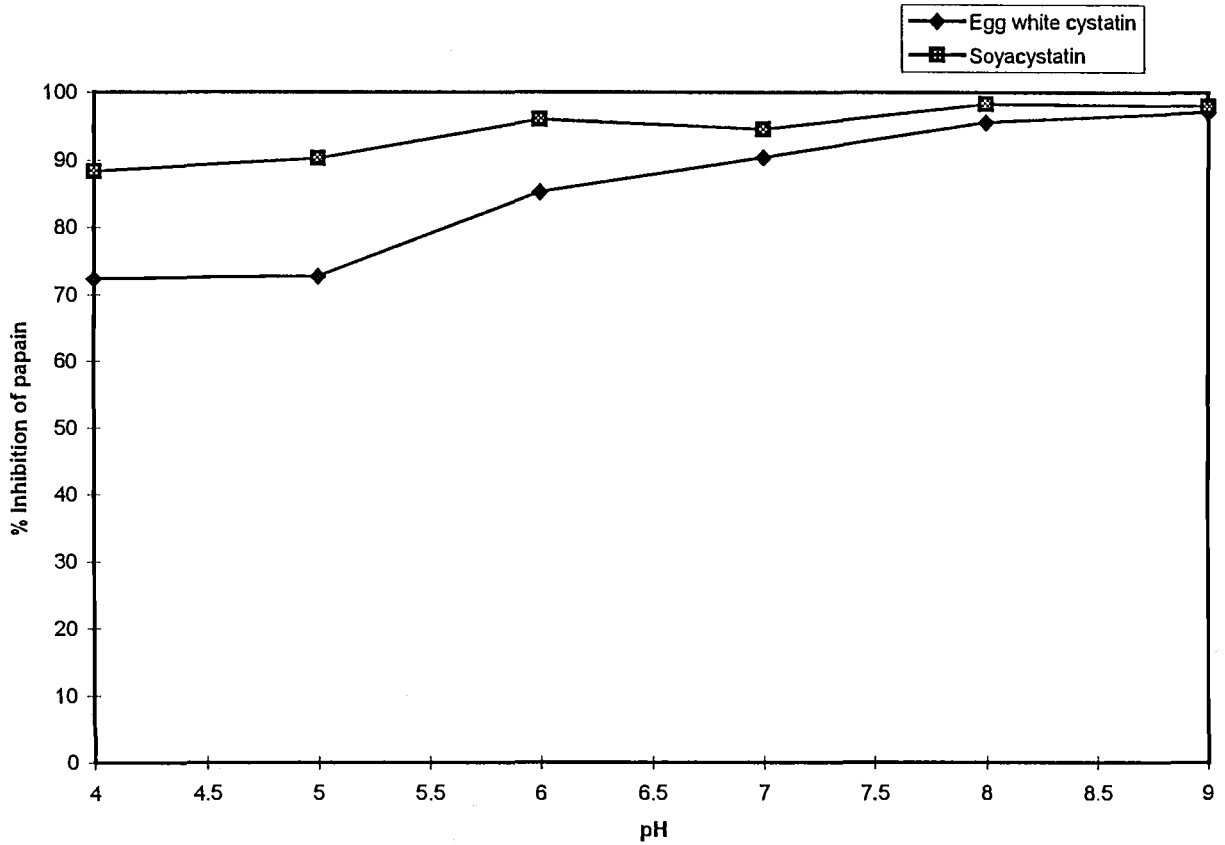

Figure 16. The percent inhibition of papain by cystatins at different pH. Relative inhibitory activity of egg white cystatin and soyacystatin was measured by analyzing residual activity of papain after reaction of papain with cystatins at 40 ^{0}C for 10 min.

Isoelectric Points of Cystatins and Papain

Isoelectric points of both cystatins and papain were determined by linear regression. Papain had an isoelectric point of 9.5 (Figures 17 and 18). This result is in agreement with the isoelectric point of papain determined by Sluyterman and Graff (1972). They found the isoelectric point of papain to be 9.6. Egg white cystatin had two proteins with identical molecular weights. They were separated into two bands by isoelectric focusing with the pI's of 5.8 and 6.6. Soyacystatin also showed two isoelectric forms on the gel at pI 5.56 and 6. Brzin et al. (1990) reported that soybean cystatin showed three major bands at pI 5.3, 5.5, and 5.9 and two minor bands at 5.4 and 8.3. Our results are in agreement with the native isoelectric points of soyacystatin.

Complex Formation of Cystatins with Proteinase

Cystatin and papain can form a complex resulting in changes in the isoelectric points (Anastasi et al., 1983). The pI of papain and egg white cystatin shifted to 8.82 and 9.2 as they formed a complex with each other (Figure 18). The complex between papain and soyacystatin was detected at pI 9.05 and 8.82 (Figure 18). Also, both cystatin complexes showed a weak band at 8.52. Figure 18 demonstrated that the complex was resolved between the papain and cystatin bands when papain and cystatin formed a complex.

Table 8 : The active concentrations of papain and cystatins used in isoelectric focusing

Enzyme and inhibitor	**Concentration (μM)**	**Amount (μg)**
Papain	24.0	2.80
Egg white cystatin	13.6	0.69
Soyacystatin	24.4	1.31

The isoelectric focusing gel shows the cystatins inhibit papain at an equimolar concentration. As seen in Table 8, the concentration of egg white cystatin used in the experiment was half that of the papain. Figure 18 shows that egg white cystatin binds papain completely. Papain was present in excessive quantity compared to egg white cystatin and was detected as both complex and unbound forms. The unbound form was found at pI 9.5. For soyacystatin, although papain was slightly oversaturated with cystatin, and a small portion of cystatin did not bind to papain and remained as a free form. The Figure 18 also showed that papain has a form which did not bind soyacystatin. According to E-64 titration, the active concentration of papain was 50%. As shown in Table 8, the result is based on the active site of papain. In other words, although 48.0 μM of papain was present, only 24.0 μM formed a complex with the cystatins. It is speculated that one half portion of papain was not active or incapable of binding cystatin.

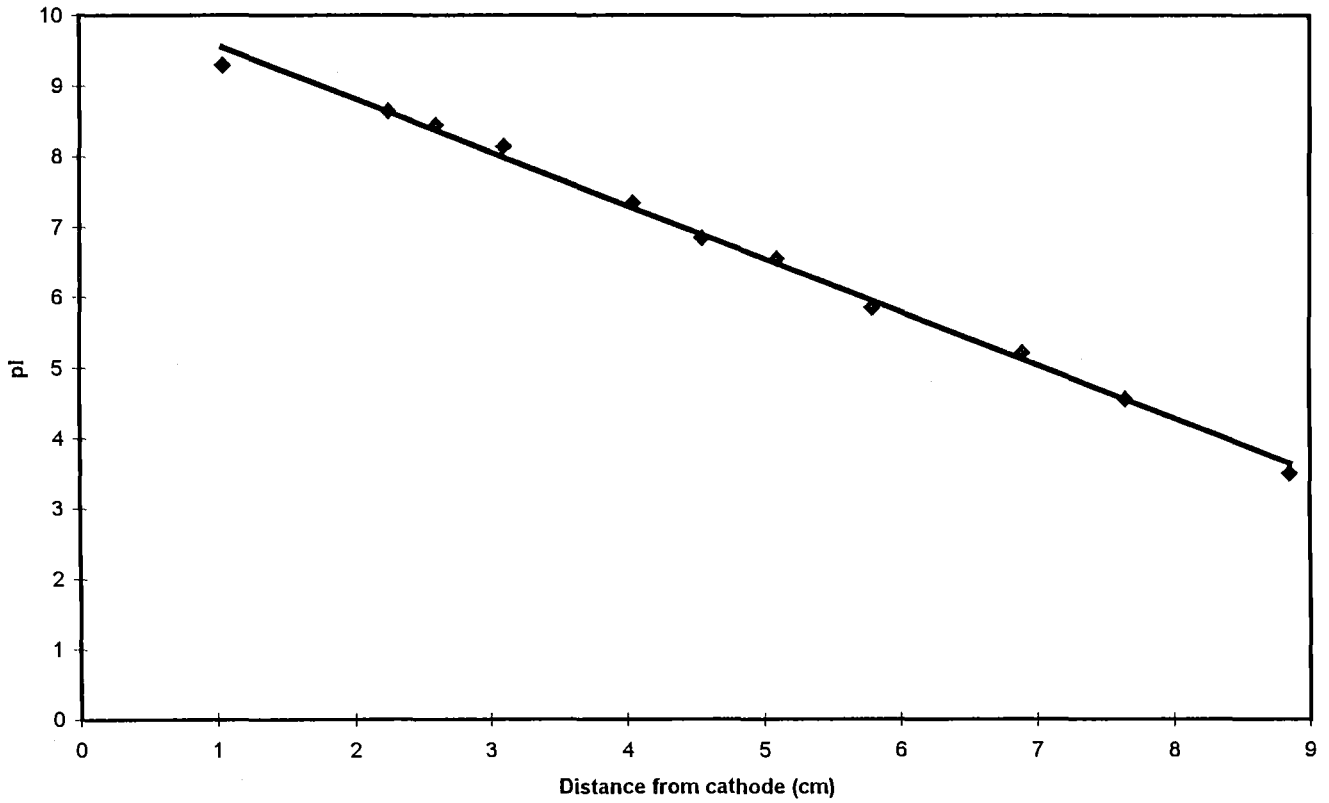

Figure 17. The linearity of pI protein standards on isoelectric focusing.

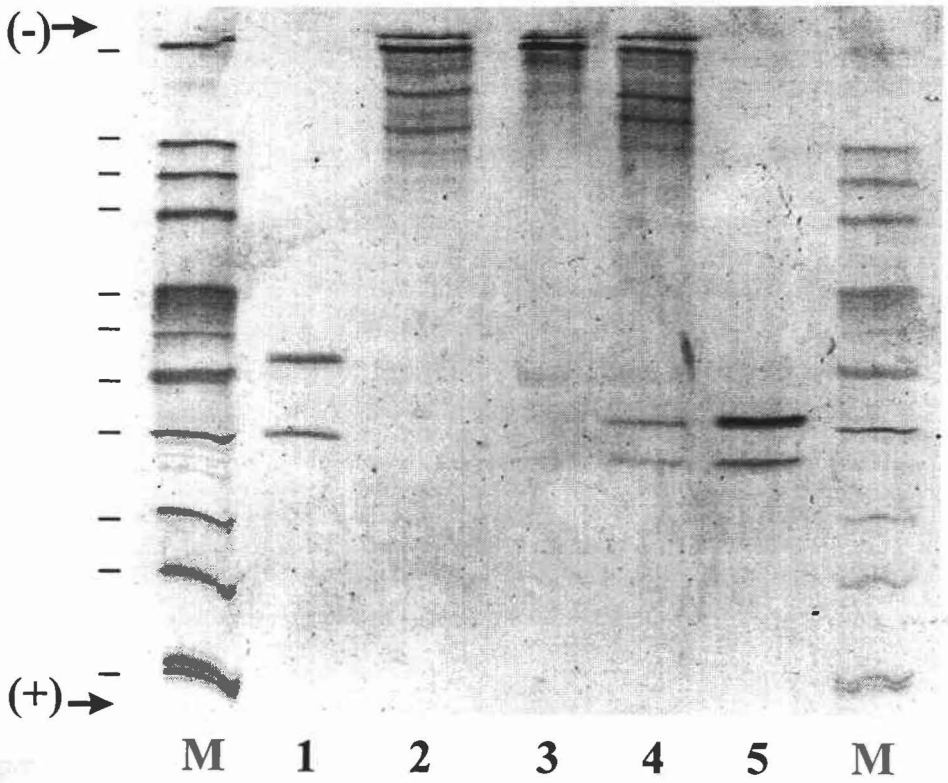

Figure 18. The complex formation of papain with soyacystatin and egg white cystatin on the IEF gel. (M) A broad range of pI standards; (1) 1.04 μg of egg white cystatin; (2) 5.6 μg of papain with 1.04 μg of egg white cystatin; (3) 5.6 μg of papain; (4) 5.6 μg of papain with 1.33 μg of soyacystatin and (5) 1.33 μg of soyacystatin;

Inhibition of Cathepsin L

Purified cathepsin L was tested against Z-Phe-Arg-Nmec at 30 ^{0}C and pH 5.5. Active site titration showed that the purified cathepsin L was 20 % active (Figure 19). The inhibition of egg white cystatin, soyacystatin and soyacystatin cell extract were tested against cathepsin L. For 50% inhibition of the cathepsin L used in the assay, 0.01877 μg of soyacystatin (Figure 20), 0.03214 μg of egg white cystatin (Figure 21) or 0.09200 μg of soyacystatin cell extract (Figure 22) was needed. As seen in Figures 19 and 20, the inhibitory activity of soyacystatin was equivalent to E-64, a powerful inhibitor of cysteine proteinase. Based on the titration curve 28.88 nM for E-64 would be needed to obtain 100% inhibition of cathepsin L, or 34.00 nM for soyacystatin (Figure 19).

Since the cathepsin L is the target enzyme in surimi products, soyacystatin gives the most promising results to prevent proteolytic degradation in surimi. Also, the soyacystatin cell extract, a crude preparation of soyacystatin, is very effective in inhibiting cathepsin L activity (Figure 20). Casein was used as a negative control. As seen in Figure 23, the effect of the casein on cathepsin L was negligible. Abe et al. (1995) purified corn cystatin II expressed in *E. coli* and they found that the corn cystatin II was also a strong inhibitor of cathepsin L. It was shown that, a cysteine proteinase inhibitor from potato inhibited cathepsin L (Rowan et al., 1990. The results presented in this study show that soyacystatin inhibits cathepsin from Pacific whiting effectively.

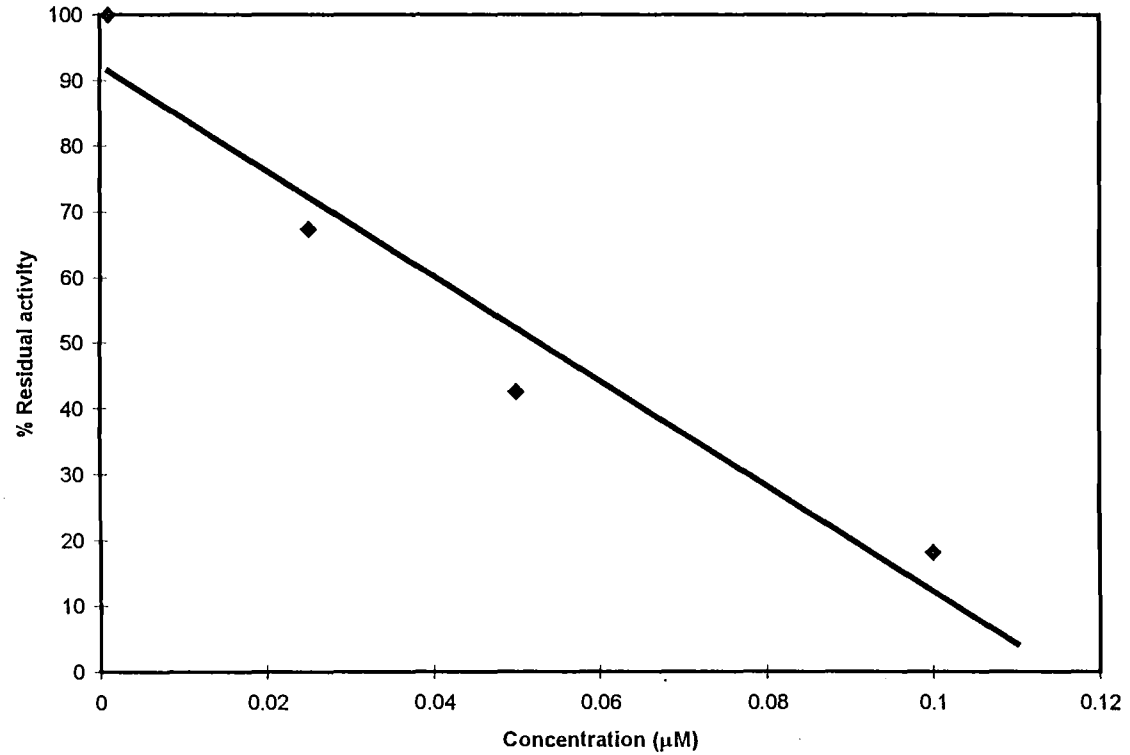

Figure 19. E-64 titration of cathepsin L. Cathepsin L was incubated with E-64 at 30 ^{0}C for 5 min, and residual activity of cathepsin L was analyzed at 30 ^{0}C for 10 min and pH 5.5 by using Z-Phe-Arg-Nmec

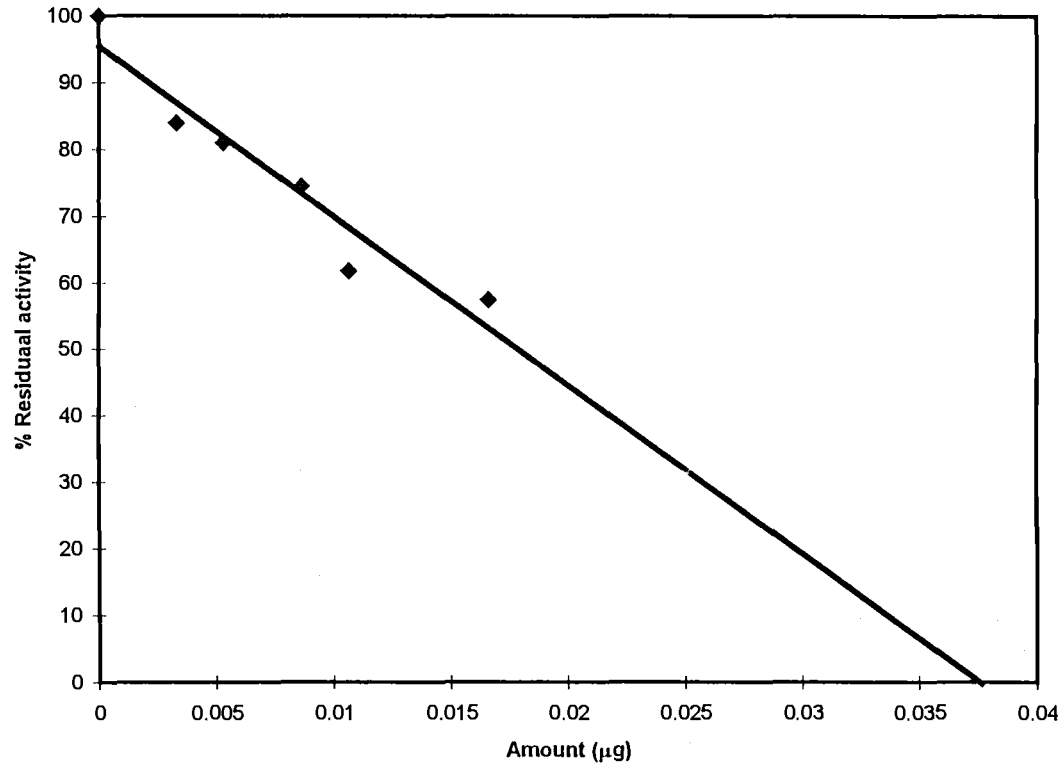

Figure 20. Inhibition of cathepsin L by soyacystatin. Cathepsin L was incubated with soyacystatin at 30 °C for 5 min, and residual activity of cathepsin L was analyzed at 30 °C for 10 min and pH 5.5 by using Z-Phe-Arg-Nmec

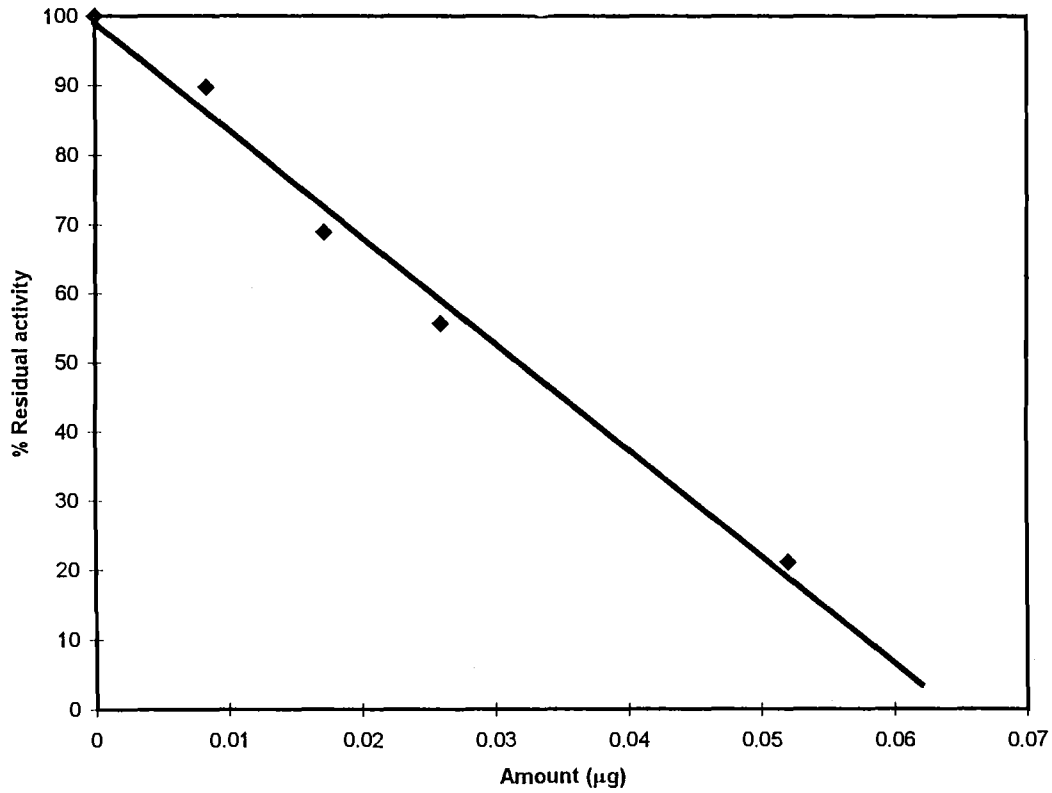

Figure 21. Inhibition of cathepsin L by egg white cystatin. Cathepsin L was incubated with egg white cystatin at 30 ^{0}C for 5 min, and residual activity of cathepsin L was analyzed at 30 ^{0}C for 10 min and pH 5.5 by using Z-Phe-Arg-Nmec

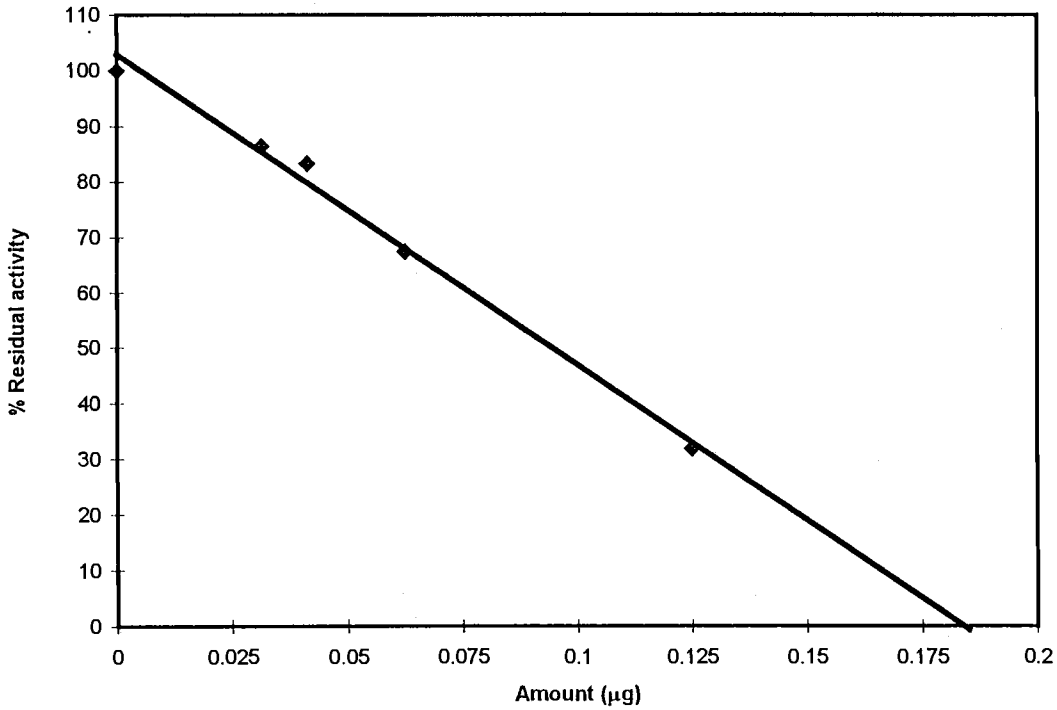

Figure 22. Inhibition of cathepsin L by soyacystatin cell extract. Cathepsin L was incubated with soyacystatin cell extract at 30 ^{0}C for 5 min, and residual activity of cathepsin L was analyzed at 30 ^{0}C for 10 min and pH 5.5 by using Z-Phe-Arg-Nmec

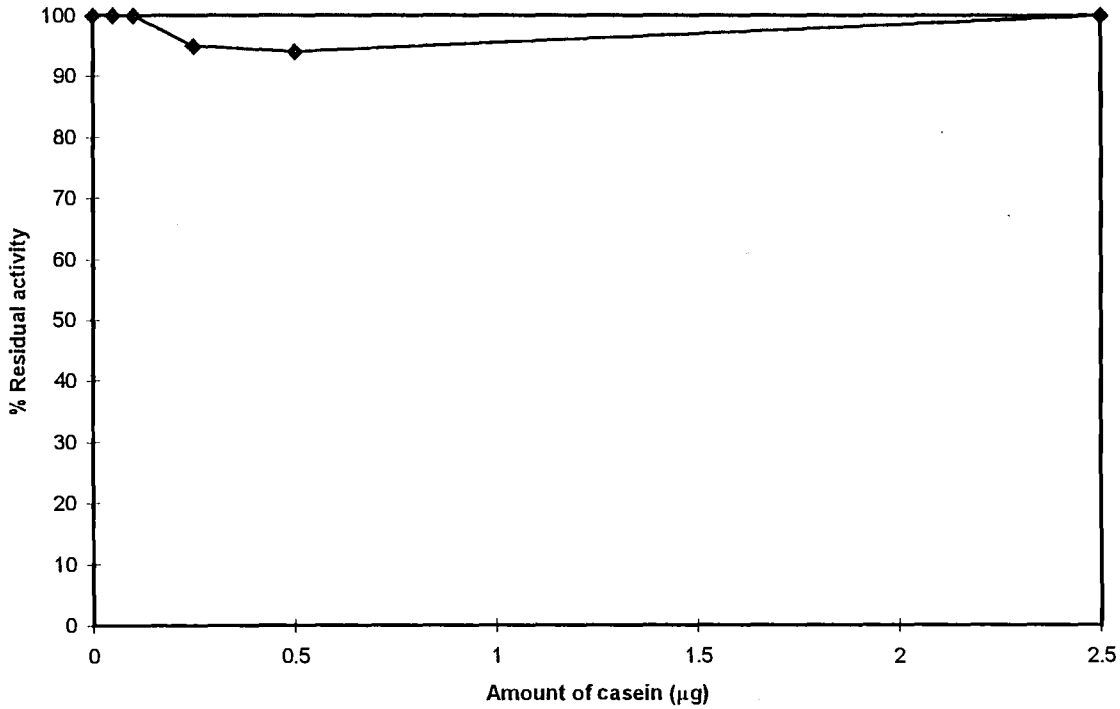

Figure 23. Inhibition of cathepsin L by casein. Casein was used as a negative control cathepsin L was incubated with casein at 30 ^{0}C for 5 min, and residual activity of cathepsin L was analyzed at 30 ^{0}C for 10 min and pH 5.5 by using Z-Phe-Arg-Nmec.

Inhibition of Autolytic Activity in Fish Muscle and Surimi

Autolytic activity in fish muscle is mainly due to cathepsin L. Cathepsin L is the major enzyme that causes textural degradation in Pacific whiting fish fillets during the conventional slow cooking process (An et al., 1994b). After the cooking of Pacific whiting surimi for 30 min at 60 ^{0}C, myosin heavy chain was completely lost (Morrissey et al., 1993). It is known that myosin degradation causes a loss of strength in surimi gel.

Parasitized Pacific whiting fillet has a high autolytic activity at 55 ^{0}C (Table 9). When 0.041% (w/w) soyacystatin cell extract was applied, a high inhibition of the autolytic activity of Pacific whiting was observed. As seen in Table 9 and Figure 24, 4% egg white inhibits only 38% of autolytic activity in the Pacific whiting muscle.

Table 9. Autolytic activity of parasitized Pacific whiting fillet with and without addition of inhibitors

Sample	**TCA soluble peptides (nmoles Tyr/min)**	**% inhibition**
Parasitized Pacific whiting fillet	4.11 $\pm$ 0.70	0
0.041 % soyacystatin cell extract	0.40 $\pm$ 0.17	90.0
0.1 mM E-64	0.13 $\pm$ 0.20	96.0
1% (w/w) BPP	3.18 $\pm$ 0.12	22.8
4% (w/w) BPP	2.18 $\pm$ 0.56	47.0
4% (w/w) Egg white	2.53 $\pm$ 1.00	38.0
4% (w/w) Casein	4.15 $\pm$ 0.95	0

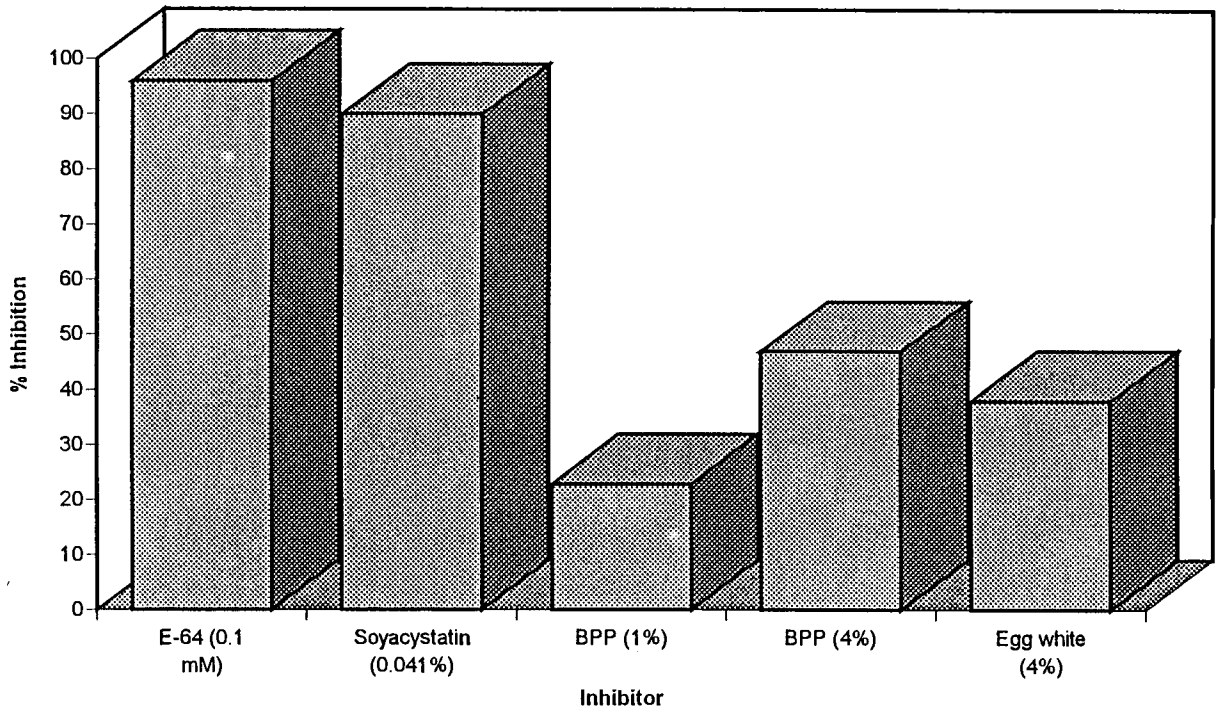

Figure 24. Percent inhibition of autolytic activity in the fish muscle by cystatins and other inhibitors. Autolytic activity of Pacific whiting fish muscle was tested at 55 ^{0}C for 30 min.

BPP has been found to be the most effective food grade inhibitor to control the protease in surimi (Morrissey et al., 1983). The proteolytic activity in muscle is higher than that in surimi. Since cathepsin B and H are removed by the washing step during surimi processing, the proteinase inhibitors in surimi deals only with cathepsin L. Therefore, BPP is very effective in inhibiting proteolysis in surimi (Figure 24), while it is not as effective as soyacystatin in preventing the proteolysis in parasitized fish fillet.

Soyacystatin cell extract was an effective inhibitor of proteolysis in parasitized Pacific whiting fish fillet. It was reported that corn cystatin inhibited the cathepsin L, H and B activity (Abe et al., 1994). Therefore, it was expected that soyacystatin would effectively inhibit proteolysis in fish muscle. Soyacystatin cell extract was more active than BPP at the 100 fold less concentration. With surimi, 1% (w/w) BPP inhibited 75.56% of autolytic activity, while 0.041% (w/w) and 0.0052% (w/w) soyacystatin cell extract inhibited 80.83% and 75.57% of autolytic activity, respectively (Table 10 and Figure 25). Soyacystatin cell extract tested in surimi at 200 fold less concentration was more active than BPP. Increasing soyacystatin concentration from 0.041% to 0.082% did not increase the percent inhibition in the autolysis of surimi (Figure 26).

Casein was used as a negative control to determine if inhibition is due to increased protein concentration. In surimi, 4% (w/w) of casein inhibited 18% of autolysis, while its inhibitory effects on the fish muscle was negligible. It was indicated that a high concentration of protein could also reduce the proteolysis by competitive inhibition although the inhibitory activity is not as specific or as high as inhibitory compounds (Weerasinghe, 1995).

Table 10. The autolytic activity of Pacific whiting surimi with and without inhibitors

Sample	**TCA soluble peptides (nmoles Tyr/min)**	**% inhibition**
Pacific whiting surimi	2.557 ± 0.018	0
0.082% soyacystatin cell extract	0.483 ± 0.023	81.11
0.041% soyacystatin cell extract	0.490 ± 0.015	80.83
0.0052% soyacystatin cell extract	0.625 ± 0.043	75.57
0.1 mM E-64	0.415 ± 0.034	83.79
1% BPP	0.624 ± 0.024	75.56
1% Egg white	1.030 ± 0.011	59.67
4% Casein	0.625 ± 0.042	18.06

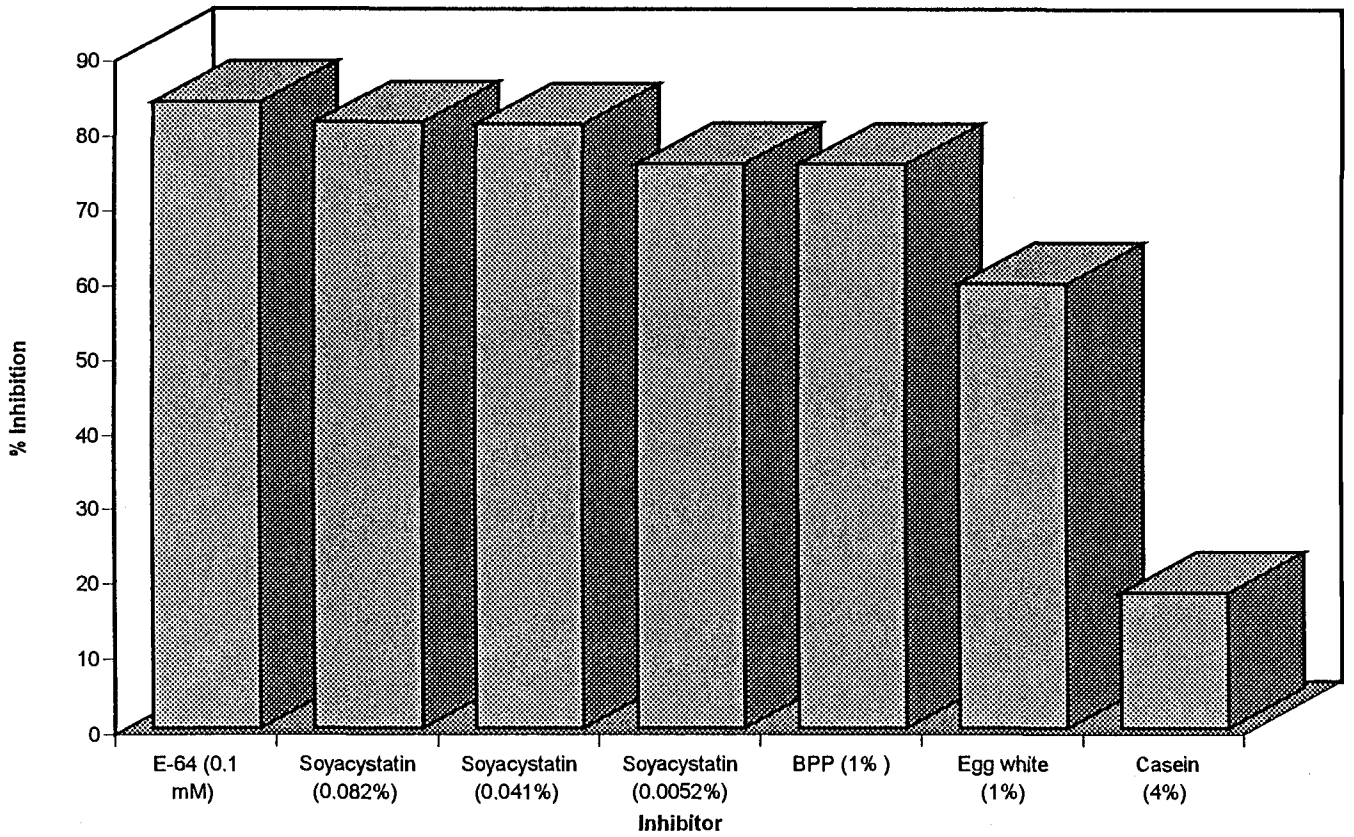

Figure 25. Percent inhibition of autolytic activity in Pacific whiting surimi by cystatins and other inhibitors. Autolytic activity of Pacific whiting surimi was tested at 55 ^{0}C for 60 min.

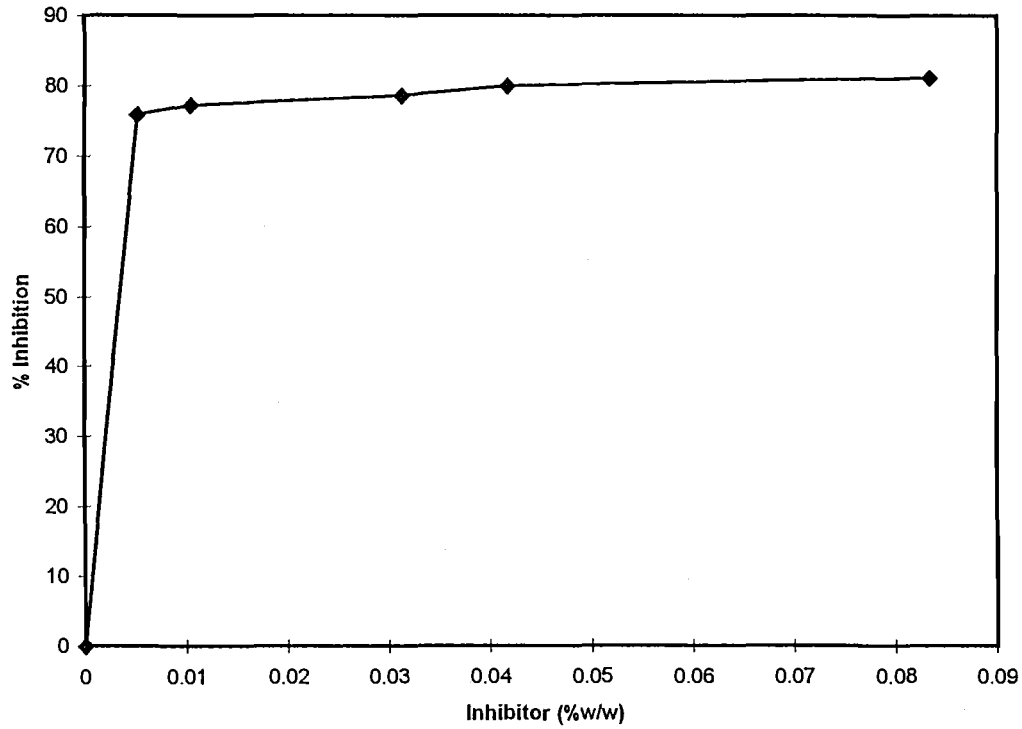

Figure 26. The inhibitory activity of different concentration of soyacystatin against the autolytic activity of Pacific whiting surimi. Autolytic activity of Pacific whiting surimi was tested at 55 ^{0}C for 60 min

CONCLUSION

Soyacystatin was purified with high yield and purity overexpressed in *E. coli*. Egg white cystatin was purified to be used as a positive control for characterization of recombinant soyacystatin and fish cystatin against cathepsin L and papain. Recombinant fish cystatin was purified but the amount of pure material was low. Recombinant fish cystatin needs harsh and long chemical treatment for renaturation. That disadvantage of the recombinant fish cystatin made it unfeasible for surimi application. Also, low yield during purification made it unsuitable for further characterization study. The inhibitory activity of recombinant fish cystatin was not as high as soyacystatin or egg white cystatin when tested against papain. Soyacystatin and egg white cystatin effectively inhibits papain, as well as the purified Pacific whiting cathepsin L.

The recombinant soyacystatin as well as egg white cystatin were stable over a broad range of pH. Even at extreme pH, such as pH 4 and 9, soyacystatin completely inhibited papain activity. On the other hand, soyacystatin was more heat sensitive than egg white cystatin. Both cystatins formed a complex with papain and the isoelectric points of the complex was found between the isoelectric points of cystatins and papain.

Soyacystatin effectively inhibits the autolytic activity of parasitized Pacific whiting muscle. Soyacystatin inhibited the autolytic activity in fish muscle and surimi to a similar degree with 100 and 200 fold less concentration than BPP. The inhibitory activity of soyacystatin was better than BPP and comparable to E-64. In conclusion, recombinant soyacystatin shows promising result as a means of controlling proteolysis during surimi processing of Pacific whiting.

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