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Kinetic properties of the two proteases, causing textural degradation of Pacific whiting (*Merluccius productus*) during heating, were compared and characterized with the synthetic substrate, Z-Phe-Arg-NMec. Pacific whiting P-I and P-II showed the highest specificity on Z-Phe-Arg-NMec, specific substrate for cathepsin L. The K_m of preactivated P-I and P-II were 62.98 and 76.02 (μM), and k_{cat}, 2.38 and 1.34 (s^{-1}) against Z-Phe-Arg-NMec at pH 7.0 and 30°C, respectively. Optimum pH stability for preactivated P-I and P-II is between 4.5 and 5.5. Both enzymes showed similar pH-induced preactivation profiles at 30°C. The maximal activity for both enzymes was obtained by preactivating the enzyme at a range of pH 5.5 to 7.5. The highest activation rate for both enzymes was determined at pH 7.5. At pH 5.5, the rate to reach the maximal activity was the slowest, but the activity was stable up to 1 hr. P-I and P-II

shared similar temperature profiles at pH 5.5 and pH 7.0 studied. Optimum temperatures at pH 5.5 and 7.0 for both proteases on the same substrate were 55°C. Significant thermal inactivation for both enzymes was shown at 75°C. Preactivated P-I and P-II displayed a similar first order thermal inactivation profile at pH 7.0. At 30 and 90°C, half lives, $t_{1/2}$, for Pacific whiting P-I were 49.50 and 0.20 min and for P-II, 32.54 and 0.18 min, respectively. The rate constant of inactivation for both proteases increased about 200-fold between two limits, 30 and 90°C. Half lives at 55°C, optimum temperature, for P-I and P-II were also determined to be 5.29 and 6.75 min. The increase in thermal inactivation rate constants independent of substrates corresponded to an activation energy for heat denaturation of 21.18 kcal/mol for P-I and 19.97 kcal/mol for P-II by Arrhenius plot. These similar kinetic properties, i.e., kinetic parameters, pH profile and thermal inactivation rate constant, suggested that Pacific whiting P-I and P-II are the same enzyme.

Kinetic Properties and Characterization of Purified Proteases from Pacific Whiting (*Merluccius productus*)

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INTRODUCTION

The commercial use of Pacific whiting (*Merluccius productus*) has been limited because of its soft texture and poor keeping quality (Erickson et al., 1983; Radtke, 1992). It is believed that the soft tissue of whiting flesh is the result of elevated proteolytic activity occurring after harvest. Extensive hydrolysis activity, by these enzymes, has been attributed to infection with Myxosporidian parasites (*Kudoa paniformis*) (Kabata and Whitaker, 1985). The abundance and low cost of Pacific whiting combined with technological advances in the use of food grade protease inhibitors permits its use as a protein base for seafood analog products.

Fish muscle proteases causing thermal degradation of texture include a heat-stable alkaline protease in menhaden (*Brevoorti tyrannus*) (Boye and Lanier, 1988), a latent serine protease in threadfin bream (*Nemipterus virgatus*) (Kinoshita et al., 1991), and cysteine proteases in Pacific whiting and arrowtooth flounder (*Atheresthes stomias*) (An et al., 1994b; Wasson et al., 1992). The thermal degradation of surimi gel texture and myosin heavy chain due to heat-stable proteases in Pacific whiting surimi was also reported by Morrissey et al. (1993).

The lysosomal cysteine proteases, cathepsins B, H, and L, have broad activities on various proteins and play a major role in the catabolism of intracellular proteins and

the breakdown of extracellular proteins (Barrett, 1977; Gerard et al., 1988; Katunuma and Kominami, 1987). Details of the physiological role of these proteases within the cell are not completely understood at this time. These proteases were also involved in meat tenderization during postmortem storage of carcasses (Mikami et al., 1987). Lysosomal cathepsins were involved in degradation of fish muscle in chum salmon (*Oncorhynchus keta*), mackerel (*Scomber japonicus*) and carp (*Cyprinus carpio*) (Hara et al., 1988; Matsumiya et al., 1989; Yamashita and Konogaya, 1991).

Proteases that cause thermal softening of swordfish (*Xiphias gladius*) and Pacific whiting muscle infected with parasites have been purified and characterized (An et al., 1994b; Konagaya, 1983; Masaki et al., 1993; Seymour et al., 1994). Swordfish proteases showed optimal caseinolytic activity between 55 and 60°C at pH 5.8 (Konagaya, 1983). Pacific whiting proteases were inhibited by sulfhydryl reagents and were active up to pH 7 (Konagaya and Aoki, 1981). Recently, these proteases have been purified and characterized as cathepsin L-like cysteine proteases with optimal activity at 55°C (Masaki et al., 1993; Seymour et al., 1994).

Cathepsin L has higher activity than other lysosomal proteases against a variety of substrates such as collagen and myofibrillar proteins (Kirschke et al., 1982; Mason et al., 1985; Okitani et al., 1980). Isolation of cathepsin L can be difficult. The enzyme tends to be unstable and forms tight complexes with its endogenous inhibitors (An et al., 1994a; Pike et al., 1992; Yamashita and Konagaya, 1992). Cathepsin L was purified and characterized from various tissues of mammals and fish (Mason, 1986; Mason et al., 1985; Yamashita and Konagaya, 1990a). However, few studies have been done on cathepsin L of fish species or the lower classes. The most specific assay for cathepsin

L activity is the fluorimetric method using Z-Phe-Arg-NMec as a substrate (Barrett and Kirschke, 1981). Cathepsin L exhibits high hydrolytic activities toward the Z-Phe-Arg-NMec with K_m of 1-5 μM and k_{cat} of 8-30 s^{-1} (Mason, 1986; Yamashita and Konogaya, 1990a). Nevertheless, cathepsin L-like cysteine proteases, isolated from parasitized Pacific whiting muscle, had lower affinity for Z-Phe-Arg-NMec than cathepsin L from mammals and chum salmon (K_m of 45 μM and k_{cat} of 4.5 s^{-1}, respectively) (Masaki et al., 1993). Cathepsin B can cleave this synthetic substrate to a lesser degree (Barrett, 1980). They can be differentiated by the inability of cathepsin L to cleave Z-Arg-Arg-NMec (Barrett and Kirschke, 1981). An inhibitor, Z-Phe-Phe-CHN_2, may also be used to distinguish cathepsin L from B.

Generally, mammalian cathepsin L is most stable between pH 4.5 and 5.5, and exhibits its maximum activity at pH 5.5 toward Z-Phe-Arg-NMec (Mason, 1986). The activity rapidly decreases above pH 7.0 (Mason, 1986). A few kinetic and thermodynamic properties of cathepsin L are documented in the literature (Machleidt et al., 1986; Turk et al., 1993). Machleidt et al. (1986) found that the inactivation rate constant of human cathepsin L, in the absence of substrate at pH 7.4, was 0.085 min^{-1}.

Cathepsin L is synthesized *in vivo* as inactive pre-pro-cathepsin L (zymogen) and subsequently processed into mature forms by multiple limited proteolysis (Mason and Massey, 1992; Nishimura et al., 1988ab). The subsequent processing of the precursor by proteolytic cleavage results in activation of the enzymatically inactive or less active procathepsin L (McDonald and Kadkhodayan, 1988). Inactivation of cathepsins at neutral pH was not effective enough to prevent extralysosomal proteolysis, when these enzymes

were released *in vivo* (Machleidt et al., 1986). Endogenous inhibitors, such as cystatins, appear to be involved in regulation of their activity (Barrett, 1987).

The two proteases (P-I and P-II) responsible for textural degradation of cooked Pacific whiting have been purified and identified as cathepsin L (Seymour et al., 1994). P-I and P-II were separated on Butyl-Sepharose hydrophobic chromatography. P-I was thought to be complexed with two low M_r components that can be dissociated by acidification, while P-II was relatively pure. The objectives of this study were to determine kinetic properties of P-I and P-II, and to study their thermal inactivation at pH 7.0.

LITERATURE REVIEW

Definition and Role of Proteolytic Enzymes in Muscle Tissue

The enzymes that can hydrolyze peptide bonds are defined as proteases. Proteases can be divided into endopeptidases (proteinases) and exopeptidases (peptidases) (McDonald, 1985). Endopeptidases hydrolyze peptide bonds of the polypeptide chain distant to the termini; whereas, exopeptidases cleave bonds adjacent to either a free α-NH_2 or α-COOH group (Barrett and McDonald, 1980; McDonald and Barrett, 1986). Endopeptidases cannot be classified based on their substrate specificity. Instead, they are divided into serine, cysteine (formerly thiol), aspartic (formerly acid or carboxyl) and metallo proteases by the nature of the essential catalytic group in the active site (Barrett and McDonald, 1980; Bird and Carter, 1980; IUB Committee on Enzyme Nomenclature, 1984). Inhibitors can help classification of endopeptidases by revealing the chemical nature of catalytic groups (Barrett, 1986). In contrast to endopeptidases, exopeptidases can be classified by the reactions that they catalyze, also by subgroups on the basis of catalytic mechanisms.

A vast number of proteases have been discovered in mammalian tissues. Liver, spleen and kidney are the richest sources of proteolytic enzymes. Although the content of proteolytic enzymes in muscle are much lower than other tissues, they play important roles in protein turnover during growth and development of animals, and in pathological conditions of muscles (Asghar and Bhatti, 1987; Barrett, 1977). The proteases also contribute to tenderness of meat during the postmortem aging process (Ouali, 1992).

Skeletal muscle that serves the function of locomotion in higher living animals is referred to as meat after postmortem aging process (Ouali, 1990). The complex process of postmortem muscle change involves metabolic, physical, and structural biological alterations in carcasses by the absence of oxygen and energy supplies, irregular release of ATP, metabolic and proteolytic enzymes and pH reduced around 5.5. The postmortem process involves at least two sets of mechanisms: 1) an enzymatic mechanism involving proteolytic enzymes, like cathepsins and calpains present in the tissue; and 2) a physicochemical mechanism by the postmortem rise in muscle osmotic pressure (Ouali, 1992). Tenderization is mainly due to the limited hydrolysis of myofibrillar proteins by endogenous muscle proteases that are active at postmortem pH (Goll et al., 1983; Robbins et al., 1979). Postmortem proteolysis can cause the disappearance of Z-disks, dissociation of actomyosin complex, destruction of connectin, denaturation of collagen, and proteolysis of myofibrils (Asghar and Bhatti, 1987; Koohmaraie, 1988a).

Proteolytic Enzymes in Muscle Cells

Intracellular protein degradation in muscle cells is mediated by at least three different proteases: calcium-dependent proteases, multicatalytic proteases, and lysosomal proteases (Bond and Butler, 1987). These proteases are located in the muscle fiber and can degrade either sarcoplasmic and/or myofibrillar proteins.

The lysosomal proteases are present in all mammalian cell types except red blood cells (Bond and Butler, 1987). The concentration of lysosome proteases are high in liver, spleen, and kidney and low in skeletal muscle. The properties of lysosomal proteases are similar among different tissues. The lysosomal enzymes are thought to involved in

protein degradation in muscle and in meat tenderization during postmortem storage of carcasses (Calkins et al., 1987; Gerard et al., 1988). Generally, the lysosomal proteases are small (20-40 kDa) and active at acidic pH. The main lysosomal enzymes are cathepsin D (aspartic protease), and cathepsins B (EC 3.4.22.1), L (EC 3.4.22.15) and H (EC 3.4.22.16) (cysteine proteases) (Barrett and Kirschke, 1981; Katunuma and Kominami, 1983). Dufour et al. (1989) have investigated the limited proteolysis of lysosomal cathepsins B, H, L, and D on fast and slow rabbit skeletal muscle myosin. Cathepsin L hydrolyzed myosin more extensively than cathepsins B and D, while cathepsin H showed negligible activity.

The calpains (EC 3.4.22.17) (calcium-dependent papain-like proteases) are a major group of calcium-dependent cysteine endopeptidases that require calcium ions for activity (Pontremoli and Melloni, 1986). All calcium-dependent proteases are optimally active at neutral pH (7.0-7.5) (Ouali, 1992). The amino acid sequence around the catalytic cysteine residue of calpain has approximately 33% homology with papain (Asghar and Bhatti, 1987). A calcium-dependent proteolytic system comprises two extensively studied enzymes with different calcium sensitivity referred to as calpain I with low Ca^{+2} requirement (μ-calpain) and calpain II with high Ca^{+2} requirement (m-calpain) (Goll et al., 1991; Kishimoto et al., 1981). Recently, another calcium-dependent enzyme different from calpain I and II was isolated from rat brain (Yoshihara et al., 1990).

Multicatalytic proteases are classified as alkaline proteases in skeletal muscle and characterized by a high molecular weight (600-700 kDa), composed of several subunits in the M_r range 20,000-35,000 (Bond and Butler, 1987). A multicatalytic protease isolated from rat skeletal muscle tissue contains active site(s) catalyzing the degradation

of Bz-Val-Gly-Arg-NMec, Suc-Ala-Ala-Phe-NMec, Z-Leu-Leu-Glu-2NNap, and [^{14}C]methyl casein (Dahlmann et al., 1985). A similar multicatalytic protease was purified from white croaker skeletal muscle (Folco et al., 1988). These proteases can be classified as trypsin-like (arg-X), chymotrypsin-like (phe-X), and peptidyl-glutamyl (glu-X) peptide bond-hydrolyzing activity (Wilk and Orlowski, 1983). Certain compounds have different effects on each of their activities. Chymostatin inhibits trypsin and chymotrypsin-like activities in the rat skeletal muscle enzyme, but activates the glu-X activity (Dahlmann et al., 1985). Multicatalytic proteases are located inside muscle cells and degrade a varity of the proteins including sarcoplasmic proteins at pH range between 7.0 and 9.0 (Dahlmann et al., 1985; Tanaka et al., 1986). Another alkaline protease found in skeletal muscle is a serine protease, not like multicatalytic proteases described above (Kinoshita et al., 1991). Attention should be given to these heat-stable alkaline proteases due to extensive thermal degradation of muscle proteins resulting in deterioration of meat quality.

Of the proteases endogenous to skeletal muscle, calcium-dependent proteases are the main proteases contributing to meat tenderization during postmortem changes (Etherington et al., 1987; Koohmaraie et al., 1988b). They are involved in the myofibrillar proteins breakdown in rat, pork, chicken, lamb, and bovine muscle (Koohmaraie et al., 1988b,c; Lowell et al., 1986; Zeece et al., 1986). On the other hand, lysosomal cathepsins and alkaline proteases are involved in thermal degradation of postmortem muscle proteins at elevated temperatures (Matsumoto et al., 1983; Yamashita and Konagaya, 1991; Yanagihara et al., 1991). Among lysosomal proteases, cathepsin D was believed to be a major protease that is involved in thermal gel degradation

(modori) (Zeece and Katoh, 1989). Currently, more attention was given to cathepsin B, H, and L in the degradation of myofibrillar proteins (Katunuma et al., 1981; Ouali et al., 1987). Hara et al. (1987) and Sakata et al. (1985) found two different types of proteases that hydrolyze hemoglobin at pH 4.0. One was possibly cathepsin B, H or L that had a broad activity on proteins and was pepstatin-insensitive, and the other was strongly inhibited by pepstatin and corresponded to cathepsin D.

On the basis of these studies, the degradation of fish muscle and meat proteins seems to be mainly caused by calcium-dependent proteases, heat stable alkaline proteases or lysosomal cathepsins. Some researchers have suggested that cooperative action of these proteases may occur to accelerate the proteolytic process (Asghar and Bhatti, 1987; Dutson, 1983).

Fish Muscle Proteases

Two different types of muscle degradation are observed depending on the temperature (Wasson, 1992). First, myofribrillar proteins of muscle are degraded during postmortem storage at refrigeration temperature. Calcium-dependent proteases located in sarcoplasm are major endogenous proteases contributing to this type of postmortem tenderness during storage (Jiang et al., 1991). The second type of degradation phenomena is found at relatively high temperature, 50°C-65°C. Thermal degradation of myofibrillar proteins is generally assumed to be caused by endogenous muscle proteases which are active at postmortem pH during heat processing. Two types of heat-stable proteases were reported in fish muscles: alkaline and acid proteases (Wasson, 1992). A heat-stable alkaline protease in white croaker was considered to cause

textural degradation at 55-75°C (Makinodan et al., 1985). The existence of these alkaline proteases in fish muscle were reported for several species, such as carp (*Cyprinus carpio*) (Iwata et al., 1973; Kinoshita et al., 1990b), white croaker (*Micropogon opercularis*) (Busconi et al., 1984; Makinodan et al., 1987), and Atlantic menhaden (*Brevoorti tyrannus*) (Boye and Lanier, 1988). These proteases are alkaline cysteine proteases and have high activity against myofibrillar proteins, especially at 60°C. They are classified as a multicatalytic protease because of its high molecular mass (M_r 43,000) with a complex subunit composition, latency of activities and multicatalytic property (Folco et al., 1988).

Another type of heat-stable alkaline protease, different from the heat-stable alkaline protease described above, was found in threadfin-bream (*Nemipterus bathybius*) (Kinoshita et al., 1991; Toyohara et al., 1990b). The authors have suggested that alkaline serine proteases might be involved in thermal gel degradation because of their strong activities on myosin heavy chain at 50-60°C. One type of heat-stable serine protease was distributed among the sarcoplasmic muscle of threadfin-bream (Toyohara et al., 1990a) and white croaker (Yanagihara et al., 1991). Another type of serine alkaline protease was purified from myofibrillar muscle of oval-filefish (*Navodon modestus*) (Toyohara et al., 1990b). Furthermore, two latent serine proteases strongly associated with myofibrils of crucian carp *(Carassius auratus cuvieri*) were found by Kinoshita et al. (1990a).

Other proteases, which contribute to thermal degradation of myofibrillar proteins in fish muscle, are acid lysosomal proteases. Siebert (1958) found that the activity of cathepsins in fish muscle was ten times greater than those of mammalian tissues. The postmortem autolysis of fish muscle by cathepsins is important in utilizing marine food

products. A major protease to cause postmortem changes is cathepsin D (Jiang et al., 1992; Makinodan et al., 1982). Extensive softening of fish muscle during heat processing was observed on arrowtooth flounder (*Atheresthes stomias*) (Greene and Babbitt, 1990), swordfish (*Xiphias gladius*) (Konagaya, 1983) and Pacific hake (*Merluccius productus*) (Masaki et al., 1993). It was generally assumed that elevated muscle degradation was associated with the infection of parasites and proteolytic activity increased during the heat processing (Hartley et al., 1993; Konagaya, 1980). As for the pathological elevation of proteolytic activity in the muscle tissue, two different mechanisms are defined. The proteases are released either by the parasites or by an immune response of the hosts (McKerrow, 1989). Cysteine proteases, found in a number of parasitic protozoa, share some characteristics with those of their hosts (North, 1992). Their enzyme characteristics and amino acid sequences are similar to those of papain, cathepsin B and L. Characterization studies on the proteases, released by parasitized fish responsible for muscle tissue softening, have been done with salmon (Bilinski et al., 1984) and Pacific whiting (Chang-Lee et al., 1989; Erickson et al., 1983). It was suggested the sulfhydryl groups were involved in the activity of the enzymes (An et al., 1994b; Bilinski et al., 1984). Morrissey et al. (1993) reported that the myosin heavy chains were substantially degraded by the proteases in the sarcoplasm of parasitized Pacific whiting (*Merluccius productus*) during precooked at 60°C. Konagaya (1983) reported that optimum temperature to hydrolyze casein for proteases causing jellification of Myxosporidia-infected swordfish muscle was between 55 and 60°C at pH 5.8. On the other hand, Konagaya and Aoki (1981) found a protease, responsible for the extensive softening of Pacific whiting muscle, was active up to pH 7.0 and inhibited by sulfhydryl

reagents. Recent investigation of these purified proteases responsible for jellification of Pacific whiting muscle showed that these proteases were a cathepsin L-like cysteine protease (Masaki et al., 1993; Seymour et al., 1994).

Increase in cathepsin levels in the salmon muscle was reported due to the duration of spawning migration not pathological conditions (Yamashita and Konogaya, 1990b). The extensive softening muscle was observed in chum salmon (*Oncorhynchus keta*) caught during spawning migration. Since the muscle exhibited high catheptic activity, it was proposed that proteolysis by these cathepsins was responsible for softening of flesh. Further studies by Yamashita and Konagaya (1991) illustrated that the most probable enzyme responsible for extensive degradation of myofibrillar proteins of salmon muscle was cathepsin L active at physiological pH, since cathepsin B and D had little proteolytic activity.

Alkaline multicatalytic proteases (Dahlmann et al., 1985; Kinoshita et al., 1990a), alkaline serine proteases (Kinoshita et al., 1990c; 1991), and acid lysosomal proteases (Yamashita and Konagaya, 1992) showed no activity on myosin-heavy chain (MHC) under physiological conditions but hydrolyzed MHC at 50-60°C. The MHC-degrading activities are latent under physiological conditions, but induced by heating in the presence of NaCl. These activities may be regulated by endogenous protease inhibitors in fish muscles (Toyohara et al., 1985; Yamashita and Konagaya, 1992). However, it is still not clear whether activation mechanism of these enzymes in physiological conditions is induced by activity of endogenous inhibitors and/or the form of zymogens.

Mammalian and Fish Cathepsin L Properties

Compared with other lysosomal proteases, cathepsin L is more active against a variety of proteins, including collagen and myofibrillar proteins. It is also capable of inactivating glucose-6-phosphate dehydrogenase and aldolase (Towatari et al., 1978).

Isolation of cathepsin L can be difficult. It is unstable and tends to form tight complexes with its endogenous inhibitors (Pike et al., 1992; Yamashita and Konagaya, 1992). Cathepsin L have been purified and characterized from various tissues of mammals: human liver, sheep and bovine liver, rabbit liver, rat liver and kidney, and rabbit skeletal muscle (Bando et al., 1986; Kirschke et al., 1977; Mason, 1986; Mason et al., 1984; Mason et al., 1985; Okitani et al., 1980). However, very little studies have been done on animals of lower classes or fish (Yamashita and Konagaya, 1990a).

Cathepsin L is classified as a papain superfamily (Kirschke and Barrett, 1987). It is the most active lysosomal cysteine protease in regard to its ability to hydrolyze azocasein (Barrett and Kirschke, 1981), elastin (Mason et al., 1985), collagen (Kirschke et al., 1982), myosin, α-actinin, and actin (Okitani et al., 1980).

The most specific assay method for analyzing activities of cathepsin L is fluorimetric method with Z-Phe-Arg-NMec as a substrate (Barrett and Kirschke, 1981). Cathepsin B also can cleave this synthetic substrate but to a less degree (Barrett, 1980). However, they can be differentiated by the inability of cathepsin L to cleave Z-Arg-Arg-NMec (Barrett and Kirschke, 1981). An inhibitor, Z-Phe-Phe-CHN_2, may also be used to distinguish cathepsin L from B. Cathepsin H is an endoaminopeptidase that can

degrade Z-Arg-NMec and catalyze the hydrolysis of unblocked amino acid derivatives such as Arg-NMec.

For nomenclature of proteases, S_n (subsite) is designated as N-terminal side of the point of cleavage of the peptide, and S'_n on C-terminal side (Schechter and Berger, 1967). The amino acid residues which bind to these subsites are referred to P_n and P'_n. The numbering is away from the site of cleavage. Specificity of cathepsin L is mostly studied by using synthetic peptide substrates (Katanuma et al., 1983), or insulin B chain (Towatari and Katunuma, 1983; Yamashita and Konagaya, 1990a) which exhibit a limited range of peptide bonds. Cathepsin L from chum salmon hydrolyzes the bonds with hydrophobic residues in P_3 and P_2, such as Phe-Val, Leu-Val, Leu-Tyr and Phe-Phe (Yamashita and Konagaya, 1990a). The bond specificity of the enzyme for insulin B chain coincided with the common cleavage sites of rat cathepsin L (Towatari and Katunuma, 1983).

Recently, cathepsin L specificity was studied using bovine β-casein (209 residues), which displays a wide diversity of peptide bonds as a substrate (Dufour and Ribadeau-Dumas, 1988). It was shown that chicken cathepsin L accepts proline residues in all positions except P_1' (mainly in P_3 and P_2' positions), and the amino acid residues in position P_2 are essentially non-polar. It hydrolyzes preferentially the Tyr-Pro pair at P_1'-P_2' positions.

Physicochemical Properties

Cathepsin L are usually found in two forms: a single-chain form of approxamate 30 kDa (Yamashita and Konagaya., 1990a) and two chain form of M_r 22-25 kDa and 5-7 kDa with the active site located on the heavy chain (Mason, 1986). The heavy chain

of cathepsin L is linked with the light chain by disulfide bonds that can be separated by reducing conditions (Mason et al., 1985).

Human cathepsin L that reveals a major band at pI 5.9 consists of multiple forms with isoelectric points in the range 5.7-6.3 (Mason et al., 1985). Similar ranges of isoelectric points were found in rat liver, rabbit liver, and chum salmon muscle cathepsin L (Kirschke et al., 1977; Mason et al., 1984; Yamashita and Konagaya, 1990a). Complete amino acid sequences of the heavy and light chains of rat, chicken, and, bovine showed high homology among species (Ritonja et al., 1988; Towatari and Katunuma, 1988; Wada et al., 1987). The heavy and light chains contained 175-176 and 42-44 amino acid residues, respectively. Comparisons of cathepsin L with plant cysteine proteases, such as papain, actinidin and aleurain, demonstrated a high degree of homology (Wada et al., 1987).

Kinetic Properties

Cathepsin L, from various species have similar kinetic properties. This enzyme exhibits high hydrolytic activity toward the synthetic substrate Z-Phe-Arg-NMec with K_m of 1-5 μM and k_{cat} of 8-30 s^{-1} (Mason, 1986; Yamashita and Konogaya, 1990a). Nevertheless, cathepsin L-like protease, isolated from parasitized Pacific whiting muscle has lower affinity to Z-Phe-Arg-NMec than cathepsin L from mammal and chum salmon. An approximate K_m reported is 45 μM, and an approximate k_{cat}, 4.5 s^{-1} (Masaki et al., 1993). Cathepsin L is strongly inhibited by microbial protease inhibitors; such as, E-64, leupeptin, chymostatin and antipain, and thiol-blocking reagents (iodoacetic acid, ρ-chloromercuribenzoate, N-ethylmaleimide and $HgCl_2$). Activity of cathepsin L is

enhanced by thiol-reducing reagents such as β-mercaptoethanol, dithiothreitol and EDTA. The rate constant of inactivation by E-64 was reported approximately 60,000 $M^{-1}\cdot S^{-1}$, and that by Z-Phe-Phe-CHN_2, a specific synthetic inhibitor for cathepsin L, was approximately 150,000 $M^{-1}\cdot S^{-1}$ (Mason, 1986).

Generally mammalian cathepsin L exhibits its maximum activity at pH 5.5 toward Z-Phe-Arg-NMec and is quite stable between pH 4.5 and 5.5. Above pH 7.0, the activity is decreased rapidly (Mason, 1986). Machleidt et al. (1986) found that the first-order inactivation rate constant of human cathepsin L was 0.085 min^{-1} at pH 7.4 in the absence of substrate. In the presence of substrate, the inactivation rate constant of human cathepsin L also followed first order (Turk et al., 1993). The substrate-independent inactivation rate constant k_{inact} was found 0.0025 min^{-1} at pH 7.4 and 37°C. Substrates may protect the enzyme from inactivation, since inactivation rate decreases with increase in substrate concentration (Machleidt et al.,1986; Turk et al., 1993) Although inactivation of cathepsin L at neutral pH was known for a long time, few little kinetic and thermodynamic investigations of this processing are documented in the literature (Barrett and Kirschke, 1981).

Regulation of Cathepsin L Activity *in Vivo*

Endogenous Inhibitor for Cathepsin L

It is well known that the lysosomal cysteine proteases are involved in intracellular protein degradation (Bohley et al., 1979). Cathepsin L is most active among the lysosomal cysteine proteases. It has highest hydrolyzing activity on proteins *in vivo* than

any other cathepsins (Barrett and Kirschke, 1981; Kirschke et al., 1982). Therefore, it can become harmful to cytosolic and extracellular proteins when this enzyme is released from the lysosomes and/or phagosomes. It is generally believed that extracellular proteolysis is regulated by enzyme inactivation at physiological extracellular pH and/or endogenous inhibitors (Machleidt et al., 1986). Machleidt et al. (1986) mentioned that pH inactivation at neutral pH was not effective enough to completely prevent extralysosomal proteolysis by released cathepsins *in vivo*. Therefore, the activity of these proteases seems to be regulated *in vivo* by endogenous inhibitors such as cystatins (Barrett, 1987).

The endogenous inhibitors can bind to the proteases tightly and reversibly. They can be subdivided into three subfamilies for lysosomal cysteine proteases: stefins, cystatins and kininogens (Turk and Wolfram, 1991). These inhibitors *in vivo* can prevent inappropriate endogenous or external proteolysis by lysosomal enzymes released into cytosol or extracellular space (Bond and Butler, 1987). Consequently, they can control intracellular or extracellular protein breakdown (Turk, 1986). *In vitro* studies, autolysis at pH 4.2 is generally used to remove endogenous inhibitors from sheep liver cathepsin L (Pike et al., 1992).

Multiple Forms of Cathepsin L

Cathepsin L can be present in multiple forms (Mason and Massey, 1992; Pike et al., 1992; Yamashita and Konagaya, 1992). Cathepsin L is synthesized *in vivo* as inactive preprocathepsin L (zymogen) and subsequently processed into its mature active forms by multiple limited proteolysis (Nishimura et al., 1988ab; Wiederanders and Kirschke, 1989). The subsequent proteolytic cleavage processing of the precusor is connected with

activation of the enzymatically inactive or less active procathepsin L (McDonald and KadKhodayan, 1988). Nishimura et al. (1988b) suggested that lysosomal cathepsin D, a major lysosomal aspartic protease, played a major role in converting procathepsin L synthesized as enzymatically inactive forms (approxamate 39 kDa) in endoplasmic reticulum into active forms (approxamate 29 and/or 25 kDa) in lysosomal compartments. *In vitro*, treatment at pH between 3.5 to 5.5 can cause limited cleavage of procathepsin L (39 kDa) into mature active forms (29 and 25 kDa) (Wiederanders and Kirschke, 1989; Mason and Massey, 1992). Inactive zymogens, less active procathepsin L, and inhibitor complex forms may be present in isolation and purification preparations. These inactive or less active forms can be fully activated by acid or heat (Fagotto, 1990; Mason et al., 1985; Mason and Massey, 1992).

Enzyme Kinetics

Enzyme-Substrate Kinetics

Michaelis and Menten (1913) proposed that the products (P) of an enzyme-catalyzed reaction arose from the breakdown of a reversible complex (ES) formed between the enzyme (E) and the substrate (S). The kinetic model is given as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Where k_1 is the rate constant for the formation of enzyme-substrate complex, and, k_{-1} and k_2 are the rate constants for the competing breakdown process that yields either a substrate or product. The constant $(k_2 + k_{-1})/k_1$ is abbreviated to K_m, the Michaelis constant. The velocity of the reaction, v_0, is defined as the rate at which the substrate

concentration decreases or alternatively the rate at which the product concentration increases. The velocity of the reaction is given by enzyme-substrate complex multiple k_2 according to the Michaelis-Menten mechanism.

$$v_0 = -d[S]/dt = d[P]/dt = k_2[ES]$$

The velocity of reaction is usually measured as the initial velocity (v_0), because v_0 simplifies the kinetic study not affected by the inhibitory products or insufficient substrates. The basic Michaelis-Menten equation can be derived by equations mentioned above:

$$v_0 = (V_{max}[S]) / (K_m + [S]) = k_2[E_t][S] / (K_m + [S])$$

$[E_t]$ is equal to $[E] + [ES]$, and V_{max}, the maximum velocity, is defined as the velocity when all the enzymes have been converted to the [ES] complex, as equal to $k_2[E_t]$. K_m is mathematically equal to the substrate concentration at which the velocity of the enzyme-catalyzed reaction reaches half the maximum value.

Determination of Kinetic Constants

The hyperbolic Michaelis-Menten equation does not allow accurate estimation of V_{max} and K_m by a direct plot of v_0 versus [S]. There are at least three transformations of the Michaelis-Menten equation that give linear forms. The most popular linear equation is given by Lineweaver and Burk (1934).

$$1/v_0 = K_m/(V_{max}[S]) + (1/V_{max})$$

A straight line can be obtained by plotting the reciprocal of the initial velocity, $1/v_0$, against the reciprocal of the substrate concentration, [S]. Another linear form of the

Michaelis-Menten equation can be made by Hanes-Woolf plot (Hanes, 1932). In this form, $[S]/v_0$ is plotted against [S].

$$[S]/v_0 = ([S]/V_{max}) + (K_m/V_{max})$$

The Michaelis-Menten equation can also be rearranged to give Eadie-Scatchard plot (Whitaker, 1972):

$$v_0 = V_{max} - (v_0 K_m)/[S]$$

$v_0/[S]$ is plotted against v_0 in this equation. The summary on determination of kinetic constants by these methods is listed in the following table.

	slope	Y intercept	X intercept
Linewear-Burk	K_m/V_{max}	$1/V_{max}$	$-1/K_m$
Hanes-Woolf	$1/V_{max}$	K_m/V_{maz}	$-K_m$
Eadie-Scatchard	$-K_m$	V_{max}	V_{max}/K_m

Temperature Dependence

The spontaneous, irreversible denaturation of enzymes at elevated temperatures is a process that is first-order with respect to enzyme.

$$-d[E]/dt = k[E];\ -d[E]/[E] = k\ dt;\ \ln[E]t = \ln[E]_0 - kt$$

Semi-logarithm of activity is plotted versus time, and the result is a straight line with a slope of -k. An empirical relationship between rate constants and temperature was formulated by Arrhenius (Stauffer, 1989).

$$\ln k = -E_a/RT + \ln A$$

Where k = rate constant; E_a = activation energy; R = gas constant; T = absolute temperature; and A = Arrhenius factor. The activation energy was determined by the slope while plotting lnk versus 1/T. If the plot of log (or ln) of activity versus time appears nonlinear, the data may fit a second-order rate law. The inactivation rate may be written as follow:

$$-d[E]/dt = k[E]^n$$

Where n is the order of the reaction. The rate of activity loss is measured at several different initial enzyme concentrations $[E_0]$. Then log (rate) is plotted versus log $[E_0]$, and the slope of this plot is n.

MATERIALS AND METHODS

Materials

Proteases, identified to be cathepsin L, were purified from the sarcoplasmic fluid of parasitized Pacific whiting (*Merluccius productus*) by the method described by Seymour et al. (1994). Two protease peaks, P-I and P-II, were separated on Butyl-Sepharose chromatography. P-I was further purified on DEAE, acidification, and Superose-12. P-II was isolated by DEAE and Superose-12 without acidification. N-Benzoyl-Phe-Arg-7-(4-methyl)coumarylamide (Z-Phe-Arg-NMec), N_{α}-benzoyl-Arg-Arg-7-(4-methyl)coumarylamide (Z-Arg-Arg-NMec), Arg-7-(4-methyl)coumarylamide (Arg-NMec), L-3-carboxy-trans-2,3-epoxypropyl-L-leucylamido(4-guanidino)butane (E-64), 7-amino-4-methylcoumarin, Brij 35 30% (w/v), and dithiothreitol were obtained from Sigma Chem. Co. (St. Louis, MO). Stock solutions of synthetic substrates, E-64, and 7-amino-4-methylcoumarin were prepared in dimethyl sulfoxide. Iodoacetic acid was obtained from Calbiochem (San Diego, CA). All chemicals were of analytical grade. Sodium acetate buffer, pH 5.5, consists of 340 mM of sodium acetate, 60 mM of acetic acid, 4 mM of disodium EDTA, and 8 mM dithiothreitol. Modified MacIlvaine buffer, pH 3 to 9, contains 0.2 M sodium phosphate, 0.1 M citrate, 1 mM of sodium azide, 4 mM of disodium EDTA, and 8 mM dithiothreitol.

Enzyme Assays

Enzyme assays were performed by the methods of Barrett and Kirschke (1981) using Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and Arg-NMec as specific substrates for

cathepsin L, B, and H, respectively (Barrett, 1980; Kirschke et al., 1982; Yamashita and Konagaya, 1990a). P-I or P-II (M.W. 28.8 KDa), 150-250 ng as determined by active site titration with E-64, were diluted with 0.1% Brij 35 to total 500 μL and mixed with 250 μL of sodium acetate buffer, pH 5.5. After one-minute incubation at 30°C, 250 μL of 20 μM of the fluorogenic substrates (Z-Phe-Arg-NMec, Z-Arg-Arg-NMec or Arg-NMec) were added to the reaction mixture. After reaction precisely for 3 min at 30°C, 200 μL of 5 mM cold iodoacetic acid was added to the reaction mixture to terminate the proteolytic reaction. The mixture was then placed on ice for at least 5 min.

The fluorescence of the released aminomethylcoumarin was determined using 0.5μM aminomethylcoumarin by excitation at 370 nm and emission at 460 nm using Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Silver Spring, MD). A unit activity was expressed as nmole of methylcoumarin released per min. All assays were performed, at least, in duplicate.

Enzyme Concentration by Active Site Titration with E-64

Active enzyme concentrations were determined by active-site titration with E-64 by the method described by Barrett and Kirschke (1981). P-I (acidified and non-acidified) or P-II, 25 μL, was preincubated with 25 μL of 25-300 nM E-64 and 50 μL of the modified MacIlvaine buffer, pH 7.0, at 30°C for 5 min, followed by dilution with 0.1% Brij 35 to the total volume of 550 μL. The mixture was added with 200 μL of sodium acetate buffer, pH 5.5, and 250 μL of 20 μM Z-Phe-Arg-NMec. After reaction exactly for 3 min at 30°C, 200 μL of 5 mM cold iodoacetic acid was added to stop the reaction.

Molarity of the enzyme active sites was determined by a linear plot of activity against E-64 molar concentrations, as described by Barrett and Kirschke (1981).

Determination of Kinetic Parameters

P-I or P-II, 150-250 ng as determined by active site titration with E-64, were diluted with 0.1% Brij 35 to total volume of 50 μL and preincubated with 50 μL of the modified MacIlvaine buffer, pH 7.0, for 5 min. Then the activity was assayed with six different concentrations of Z-Phe-Arg-NMec ranging from 15 to 40 μM (final concentration in the mixture) in sodium acetate buffer, pH 5.5, as described in "Enzyme Assays". Values of K_m and V_{max} for enzymes were derived from the Hanes-Woolf plot by the method of Wilkinson (1961). Values of k_{cat} were calculated from the equation: $V_{max}/[E] = k_{cat}$, where [E] stands for the active enzyme concentration.

pH-Induced Preactivation of Enzymes

P-I or P-II, 150-250 ng as determined by active site titration, were diluted with 0.1% Brij 35 to the total volume of 50 μL and incubated with 50 μL of the modified MacIlvaine buffer, pH 3.5- 7.5, for intervals ranging from 0 to 60 min at 30°C. Then this enzyme mixture was added with an assay cocktail prewormed to 30°C. The cocktail solution contained 450 μL of 0.1% Brij 35, 200 μL of sodium acetate buffer, pH 5.5, and 250 μL of 20 μM Z-Phe-Arg-NMec. The activity was measured by reaction for 3 min at 30°C.

Effect of pH

P-I (non-acidifed and acidified) or P-II, 150-250 ng as determined by active site titration, were diluted to 50 μL with 0.1% Brij 35. The diluent was added with 50 μL of the modified MacIlvaine buffer, pH 3-9, and preincubated for 30 min at 0°C. The enzyme mixture was added with an assay cocktail containing 450 μL of 0.1% Brij 35, 200 μL of sodium acetate buffer, pH 5.5, and 250 μL of 20 μM Z-Phe-Arg-NMec prewarmed to 30°C. The activities of the enzymes were measured after reaction for 3 min at 30°C.

The pH profile of preactivated enzymes was determined by incubating enzymes in 50 μL of the modified MacIlvaine buffer, pH 7.0, for 5 min at 30°C. The preactivated enzymes were added with 200 μL of the modified MacIlvaine buffer, pH 3.5-9, and further incubated at 30°C for 1 hr for a stability test. Then 100 μL of enzyme mixture was added with an assay cocktail made of 400 μL of 0.1% Brij 35, 250 μL of sodium acetate buffer, pH 5.5, and 250 μL of 20 μM Z-Phe-Arg-NMec prewarmed to 30°C. The enzyme activity was analyzed by reaction for 3 min at 30°C.

Effect of Temperature on Activity

P-I or P-II, 150-250 ng as determined by active site titration, were diluted to 500 μL with 0.1% Brij 35 and added with 250 μL of the modified MacIlvaine buffer, pH 5.5 and 7. Then the enzyme activity was determined with against 250 μL of 0.1 mM Z-Phe-Arg-NMec as a substrate at various temperatures from 0 to 75°C.

Thermal Inactivation

A spontaneous first-order thermal inactivation of enzymes was determined by the method described by Stauffer (1989). Enzymes were diluted with water and preactivated in the modified MacIlvaine buffer, pH 7.0, at 30°C for 5 min. Aliquots of preactivated enzyme were incubated at different temperatures from 30 to 90°C in the modified MacIlvaine buffer, pH 7.0. Aliquots of the solution were cooled quickly on ice at various intervals, 0-60 min, and then the activity was measured against Z-Phe-Arg-NMec in sodium acetate buffer, pH 5.5 at 30°C.

The rate constant of inactivation, k_{inact}, was determined from the slope by plotting semilogarithm (ln) of residual activity (%) against the time. The half-life is given by ln $2/k_{inact}$. Activation energy was calculated by Arrhenius plot of inactivation rate constants against absolute temperature at pH 7.0. The Arrhenius equation is given as $\ln k_{inact} = - Ea/RT + \ln A$.

RESULTS

Activity on Peptidyl-NMec Substrates

Pacific whiting proteases, P-I and P-II, had high hydrolytic activities against the substrate Z-Phe-Arg-NMec but negligible against Z-Arg-Arg-NMec and Arg-NMec at 30°C (Table 1). The activities of P-I and P-II at pH 7.0 were about two fold that at pH 5.5. The activities on Z-Phe-Arg-NMec for both enzymes at pH 3.0 were negligible. This evidence indicate that both proteases are relatively free of closely related enzymes, such as cathepsin B.

The K_m and k_{cat} of preactivated P-I were determined as 62.98±5.66 (μM) and 2.38±0.22 (s^{-1}), respectively. The Km and kcat of preactivated P-II against Z-Phe-Arg-NMec were determined to be 76.02 ± 3.00 (μM) and 1.34 ± 0.06 (s^{-1}), respectively (Table 2). Studies of cathepsin L from human, ox, sheep, rabbit and rat (Mason, 1986) have shown that an important characteristic is high affinity against Z-Phe-Arg-NMec, as shown by low K_m of 1-4 μM. On the other hand, closely related enzymes to cathepsin L such as cathepsin B has low affinity with Z-Phe-Arg-NMec as shown by a K_m value of 75 μM (Mason et al., 1984). Pacific whiting P-I and P-II have low affinity for Z-Phe-Arg-NMec compared with mammalian cathepsin L. Nevertheless, Pacific whiting P-I and P-II did not hydrolyze Z-Arg-Arg-NMec and Arg-NMec that are highly selective for activities of cathepsin B and H, respectively. The Pacific whiting protease, identified to be cathepsin L-like by Masaki et al. (1993), also showed low affinity with Z-Phe-Arg-NMec substrate (K_m of 42- 47 μM), which is similar to those of Pacific whiting P-I and P-II.

Effect of pH

Maximal activities of non-preactivated P-I and P-II against Z-Phe-Arg-NMec at 30°C were found at pH 7.0, when activities were analyzed after incubating both enzymes in the absence of substrate at 0°C for 30 min (Fig. 1). The residual activities of Pacific whiting P-I and P-II were above 100% with high activities between pH 6 to 8 after incubation in the various pH buffers for 30 min at 0°C. Therefore, it was assumed that Pacific whiting P-I and P-II were activated by incubation for 30 min in most pH range. The higest activation was observed at pH 7.0.

pH profile of preactivated Pacific whiting P-I and P-II was also studied by incubating preactivated enzymes for 1 hr at 30°C (Fig. 2). It was shown that both P-I and P-II were most stable at acidic conditions, pH 4.5-5.5. At this pH range, Pacific whiting P-I and P-II did not lose activity even after 1 hr incubation. At pH 7.0, the activity of P-I and P-II decreased to about 50% after 1 hr incubation. A rapid loss in activity was observed above pH 7.0. The activity of Pacific whiting proteases decreased to about 25% at pH 7.5 after 1 hr incubation. Mammalian cathepsin L is known to be stable in the pH range 4.5-5.5 and rapidly inactivated above pH 7 (Mason, et al., 1985; Mason, 1986). Pacific whiting proteases have same pH profile as mammalian cathepsin L which is stable at pH 4.5 to 5.5.

pH-Induced Preactivation

Activation pattern of Pacific whiting proteases is shown for pH ranges of 3.5 to 7.5 for periods of 0 to 60 min at 30°C (Fig. 3). Maximal activity for both P-I and P-II

was obtained by preactivating the enzyme at pH 5.5 to 7.5. Once enzymes were activated, they were highly subjected to inactivation. The rate of preactivation at pH 7.5 is the highest (approxamate 1 min), followed by the most rapid inactivation rate of the enzymes. At pH 5.5, the rate of activation was the slowest (approxamate 30 min), but the activity was most stable up to 1 hr. At pH 7.0, activities of both proteases reached the maximal activity in 5 min followed by a substantial decline. At pH 3.5 and 4.5, activity rapidly reached a plateau and the maximal activities were only 50 and 80% of pH 5.5, respectively. Both P-I and P-II showed a similar pH-induced preactivation profile. The pH-induced preactivation profile may explain why Pacific whiting P-I and P-II showed a broad peak of activity extending to the alkaline region shown in Fig. 1. This result suggests that Pacific whiting P-I and P-II needs to be preactivated prior to activity assay in order to measure the true activity. Without the preactivation, the activity may only reflect the activation rate on the test conditions. Therefore, for further experiments, P-I and P-II were preactivated at pH 7.0 for 5 min prior to activity assays.

Effect of Temperature on Activity

The optimum temperatures for both Pacific whiting P-I and P-II were shown to be 55°C (Fig. 4). The optimum temperatures for Pacific whiting proteases on Z-Phe-Arg-NMec were the same as for myosin degradation in Pacific whiting surimi (Chang-Lee et al., 1989). The protease responsible for jellification of the Swordfish infected with Myxosporidia (*Chloromyxum musculoliquefaciens*) also had a similar temperature optimum between 55°C and 60°C against casein (Konagaya, 1983). P-I and P-II both showed the higher activities against Z-Phe-Arg-NMec at pH 5.5 than pH 7.0 in the

temperature range from 45 to 65°C. Below 45°C, there was no significant difference in activity between pH 5.5 and pH 7.0. Pacific whiting proteases were substantially inactivated above 65°C at both pHs studied. Almost complete inactivation of the enzymes was observed at 75°C. The dramatic decrease in activity against Z-Phe-Arg-NMec at high temperatures above 75°C was also shown against casein (Seymour et al., 1994). Since the activities of Pacific whiting proteases were substantially reduced above 75°C, rapid cooking such as deep-fat frying and Ohmic heating (Patashnik et al., 1982; Biss et al., 1989) can be a good approach for inactivating proteases. By rapid heating, the thermal inactivation of enzyme may be achieved before proteolysis sets in and degrades fish muscle proteins.

Thermal Inactivation

Thermal inactivation of Pacific whiting P-I and P-II was investigated for various incubation times and temperatures ranging from 30 to 90°C at pH 7.0 (Fig. 5). Both Pacific whiting P-I and P-II showed a first order thermal inactivation. Half-lives ($t_{1/2}$) of Pacific whiting P-I for two temperature limits, 30 and 90°C, were calculated to be 49.50 and 0.20 min, respectively; and those of P-II to be 32.54 and 0.18 min, respectively (Table 3). The rate constant of inactivation for both proteases increased about 200-fold by raising temperature from 30 to 90°C. Half lives at 55°C for P-I and P-II were calculated to be 5.29 and 6.75 (min), respectively. The increase in inactivation rate independent of substrate corresponded to an activation energy for heat denaturation of 21.18 Kcal/mol (88.60 KJ/mol) for P-I and 19.97 Kcal/mol (83.53 KJ/mol) for P-II as determined by Arrhenius plot (Fig. 6).

Effect of acidification on P-I

Active site titration with E-64 using Z-Phe-Arg-NMec as a substrate revealed that acidified P-I of Pacific whiting titrated completely with E-64 (Fig. 7). By contrast, only 55% of the Pacific whiting non-acidified P-I activity titrated with E-64 (Fig 7). This results indicated that non-acidified P-I exists in a complex form with an inhibitor, which is active against Z-Phe-Arg-NMec, but inaccessible to E-64. Similar result is shown for sheep liver cathepsin L which was proposed to be present in cystatin-complex forms (Pike et al., 1992). Acidification at pH 4.5 can have a significant effect on activity by dissociating the complex to expose the active site of enzymes (Fig. 8). The pH optimum for non-acidified P-I against Z-Phe-Arg-NMec was pH 4.5 which was shifted to pH 7 after acidification (Fig. 8). pH optimum at 7 reflects the more accurate value for the proteases, since the proteases are relatively free of inhibitor after acid treatment. A similar pH optimal profile, pH 3.5-3.9 and 7.1-7.2, for parasitized Pacific whiting on hemoglobin was also reported by Erickson et al. (1983).

DISCUSSION

The thiol proteases are often isolated in an inactive form. It is generally accepted that protease activity in the cells is strictly regulated by the existence of inactive zymogens as in the rat liver microsomal fractions (Nishimura et al., 1988b) or by endogenous inhibitors as found in Chum salmon and sheep liver (Pike et al., 1992; Yamashita and Konagaya, 1992).

Yamashita and Konagaya (1992) proposed that the complex found in Chum salmon consisted of the 37 kDa form of precursor of cathepsin L, 30 kDa form of cathepsin L and the 15 kDa endogenous cysteine protease inhibitor based on SDS-PAGE analysis and the activation of enzyme by acidification. Fagotto (1990) showed that the egg cathepsin L is stored in the yolk as a proenzyme which is activated by partial proteolysis at low pH. Cathepsin L was first synthesized as a 39 kDa protein and subsequently processed into the mature forms of 30 and 25 kDa in the cell (Nishimura et al., 1988a). Nishimura et al. (1988b) reported that procathepsin L was first synthesized as enzymatically inactive forms in endoplasmic reticulum and converted into active forms by cathepsin D in lysosomal compartments. The major excreted protein (MEP) from a transformed mouse fibroblast cell, later identified as a precursor form of cathepsin L (Mason et al., 1987), is a 39 kDa acid-activatable glycoprotein and can converts to lower molecular weight forms of 29 and 20 kDa which are localized in the Golgi complex and lysosomes (Gal and Gottesman, 1986).

In previous studies, Pacific whiting P-I and P-II were shown to have the same molecular weights of 28.8 KDa, and isoelectric points of 4.99, and identified as cathepsin

L (Seymour et al., 1994). Based on kinetic properties studied in this paper, it was suggested that Pacific whiting P-I and P-II are a same enzyme. Procathepsin L, contamined with inhibitors complex (non-acidified P-I) might have been processed to a mature form (P-II) *in vitro* by heating during purification. The mature form (P-II) is presumably separated from procathepsin L inhibitor complex (non-acidifed P-I) by different hydrophobic interaction on butyl sepharose chromatography.

Table 1. Activity of Pacific whiting P-I and P-II against synthetic substrate at 30°C

pH	Z-Phe-Arg-NMec		Z-Arg-Arg-NMec		Arg-NMec	
	P-I	P-II	P-I	P-II	P-I	P-II
	(U/nmole of active site)					
3.0	0 .69 ± 0.19	0.25 ± 0.00	0.00 ± 0.00	0.00 ± 0.07	0.00 ± 0.01	0.06 ± 0.01
5.5	3.84 ± 0.83	2.09 ± 0.37	0.00 ± 0.00	0.03 ± 0.04	0.00 ± 0.01	0.15 ± 0.06
7.0	6.45 ± 0.40	4.75 ± 0.37	0.01 ± 0.00	0.04 ± 0.04	0.06 ± 0.08	0.23 ± 0.05

Table 2. Kinetic constants for hydrolysis of Z-Phe-Arg-NMec by Pacific whiting preactivated P-I and P-II at 30°C

Enzyme	K_m (μM)	$k_{cat.}$ (s^{-1})
P-I	62.98 ± 5.66	2.38 ± 0.22
P-II	76.02 ± 3.00	1.34 ± 0.06

Table 3. Effect of temperature on rate constant of inactivation of Pacific whiting preactivated P-I and P-II against Z-Phe-Arg-NMec

T(°C)	P-I		P-II	
	k_{inact} (min^{-1}) (mean ± S.D.)	$t_{1/2}$ (min)	k_{inact}	$t_{1/2}$
30	0.014 ± 0.002	49.50	0.021 ± 0.004	32.54
45	0.037 ± 0.007	18.68	0.047 ± 0.007	14.81
55	0.130 ± 0..021	5.29	0.103 ± 0.011	6.75
65	0.406 ± 0.051	1.71	0.313 ± 0.024	2.21
75	1.225 ± 0.551	0.57	1.271 ± 0.126	0.55
90	3.484 ± 1.177	0.20	3.914 ± 1.971	0.18

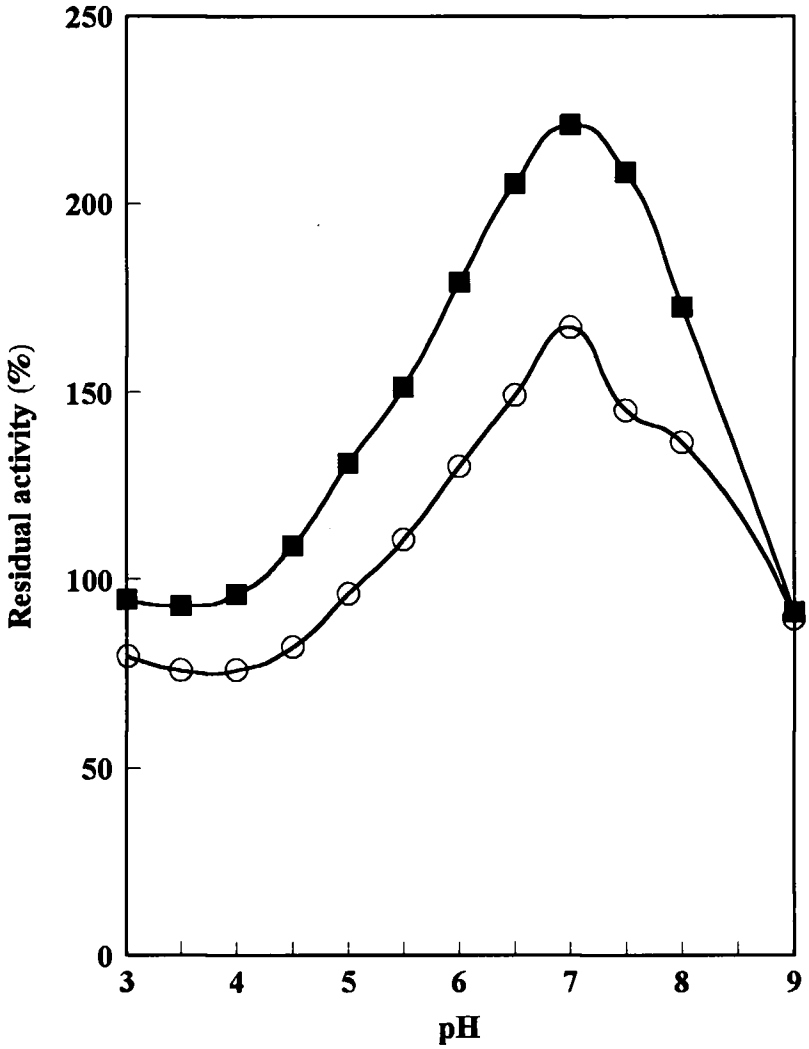

Fig. 1. pH stability of Pacific whiting P-I (-■-), and P-II (-○-) after incubation for 30 min at 0°C

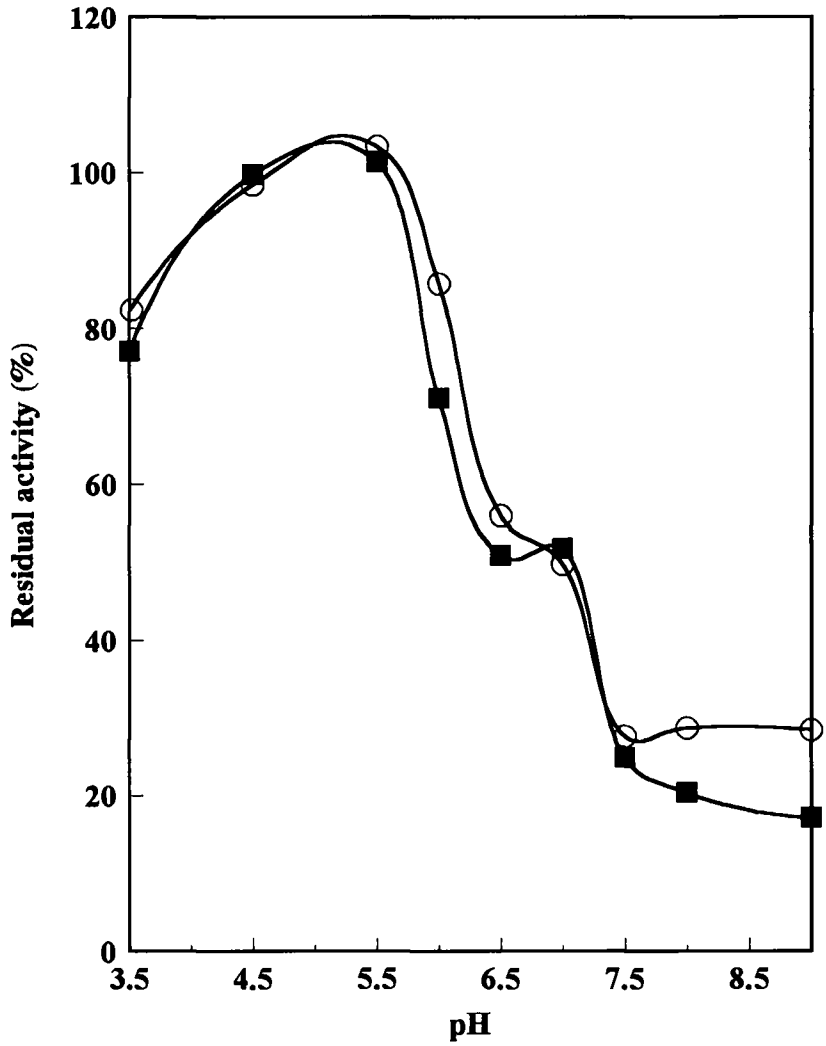

Fig. 2. pH stability of Pacific whiting preactivated P-I (-■-) and P-II (-○-) after incubation for 1 hr at 30°C

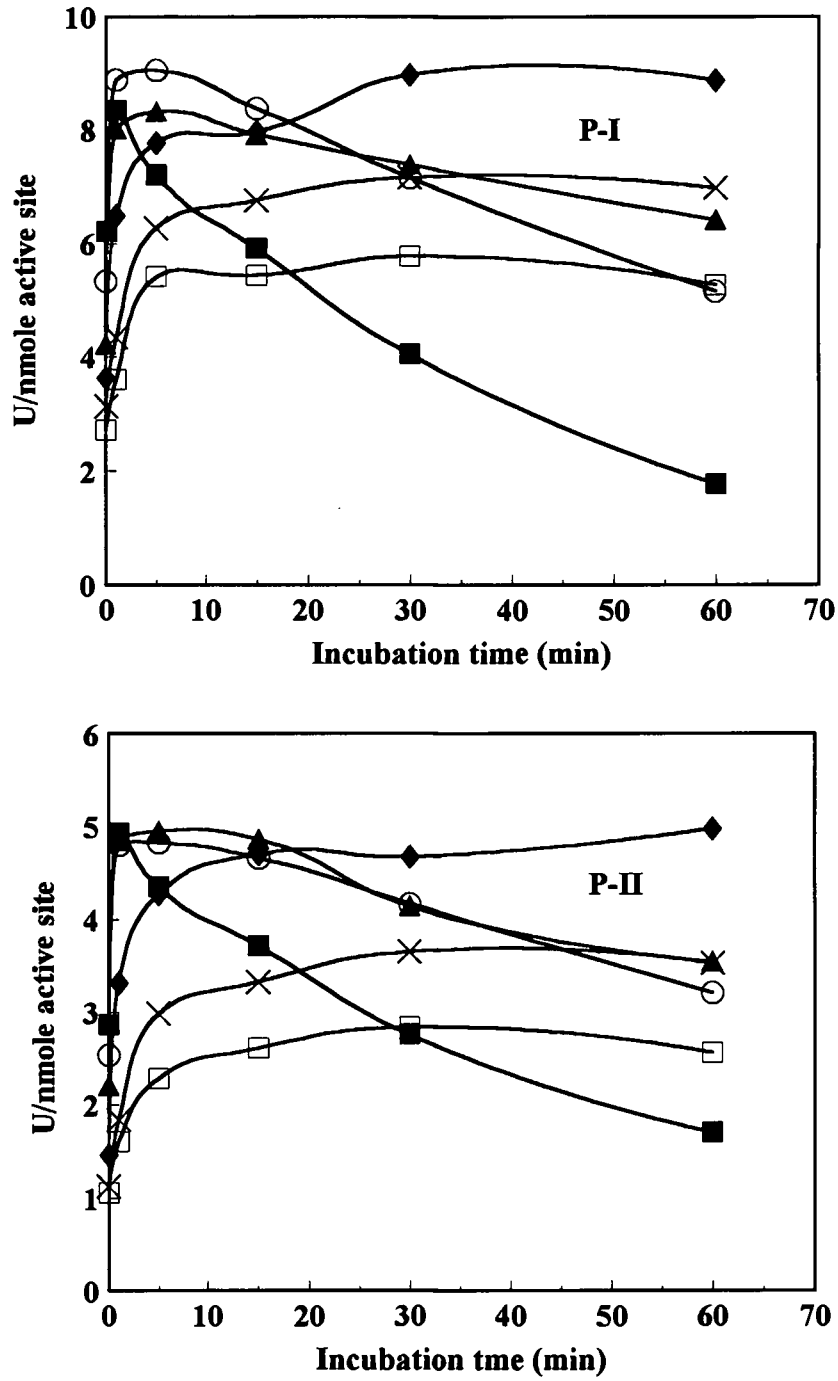

Fig. 3. pH-induced preactivation of Pacific whiting P-I and P-II (-□- = pH 3.5; -×- = pH 4.5; -◆- = pH 5.5; -▲- = pH 6.5; -○- = pH 7.0; -■- = pH 7.5) for periods of 0 to 60 min at 30°C

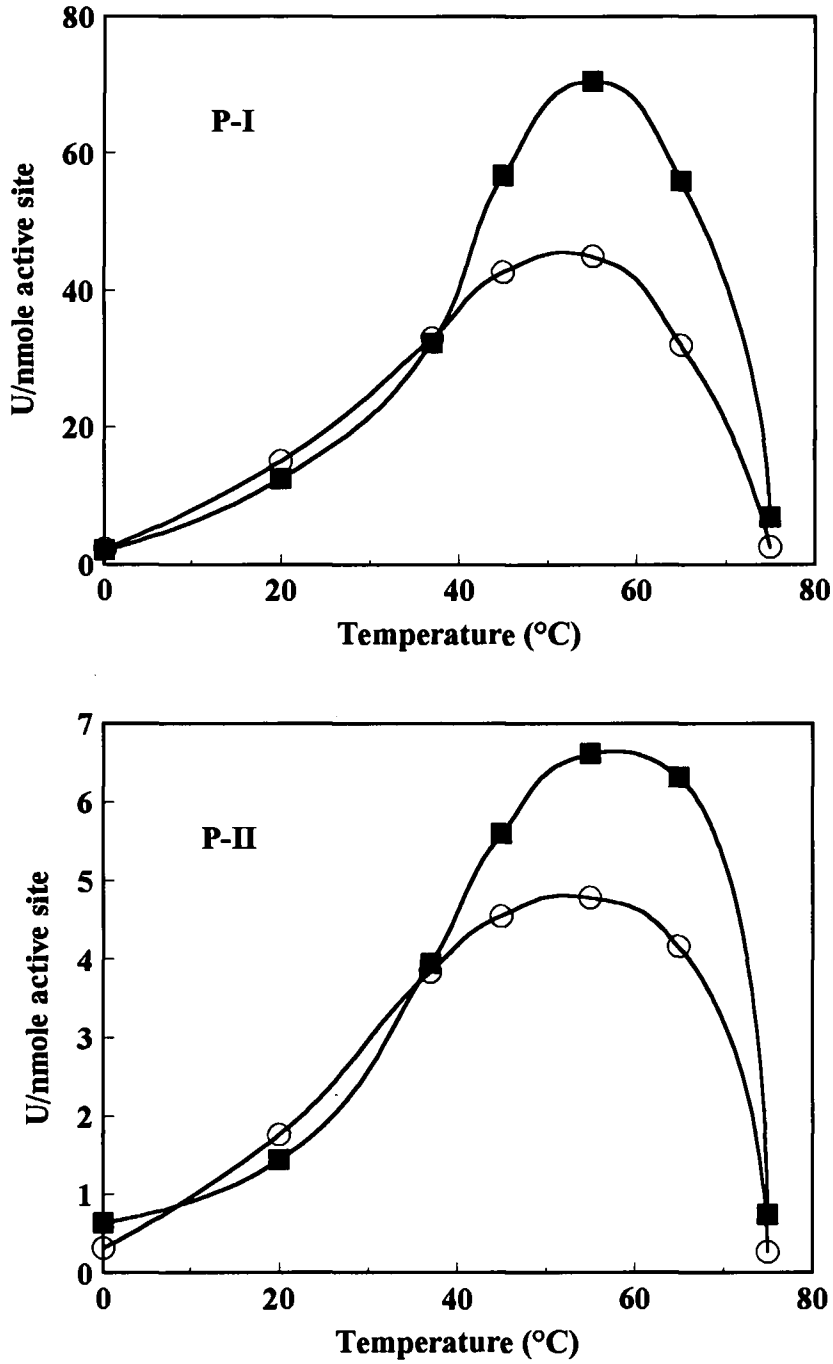

Fig. 4. Effect of temperature on the activity of Pacific whiting P-I and P-II at pH 5.5 (-■-) and pH 7 .0 (-○-)

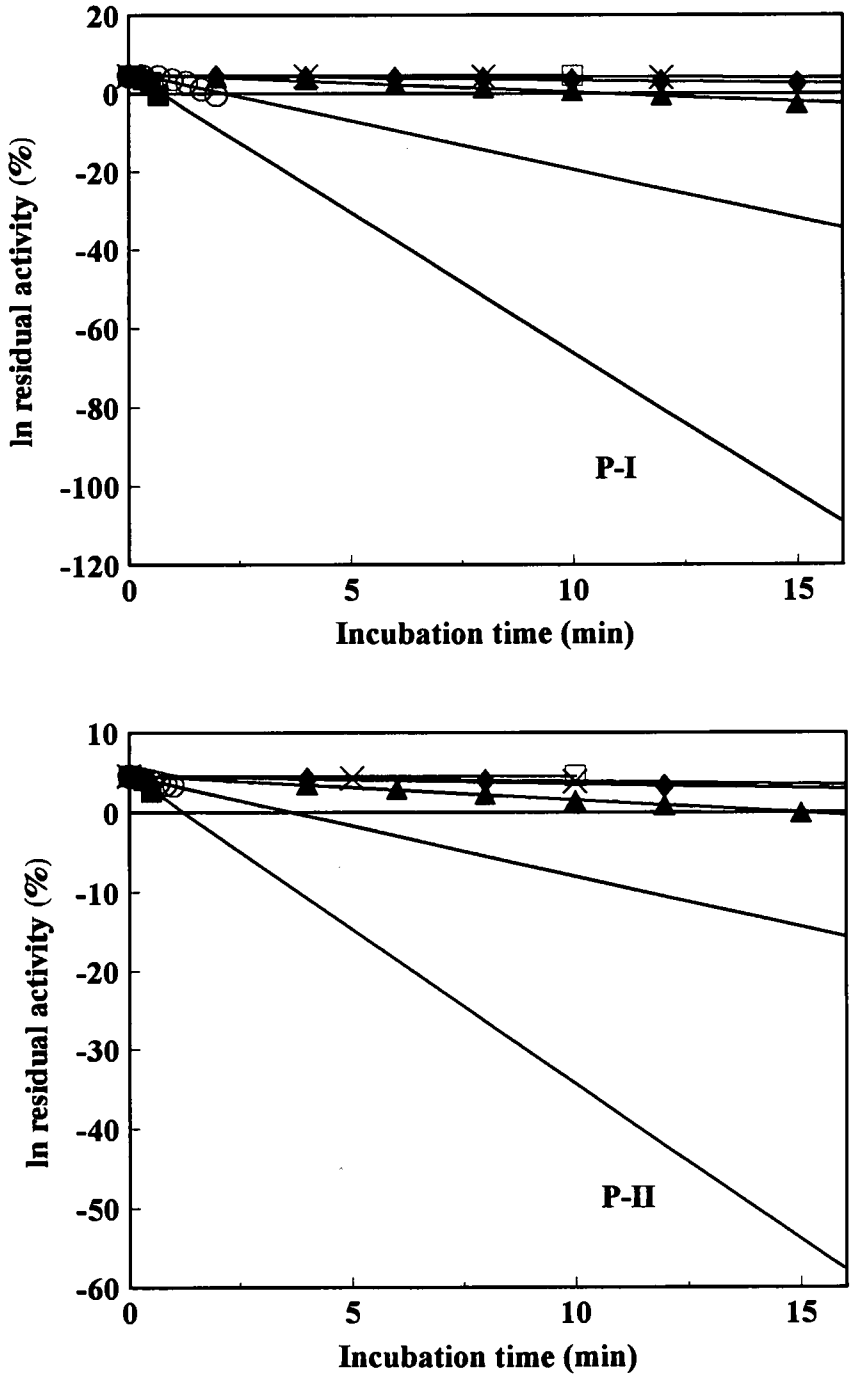

Fig. 5. Thermal inactivation of Pacific whiting preactivated P-I and P-II at various temperatures (-□- = 30°C; -×- = 45°C; -◆- = 55°C; -▲- = 65°C; -○- = 75°C; -■- = 90°C) at pH 7.0

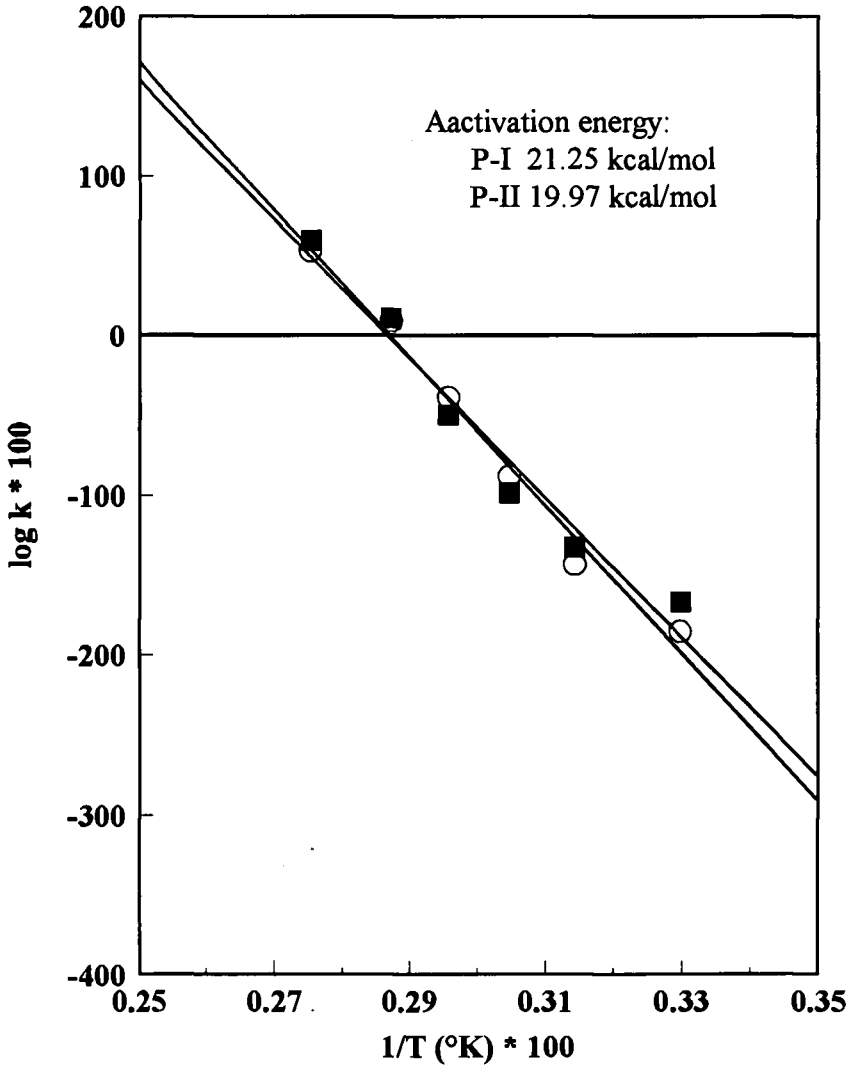

Fig. 6. Arrhenius plot for the thermal inactivation rate constants of Pacific whiting preactivated P-I (-○-) and P-II (-■-) at pH 7.0

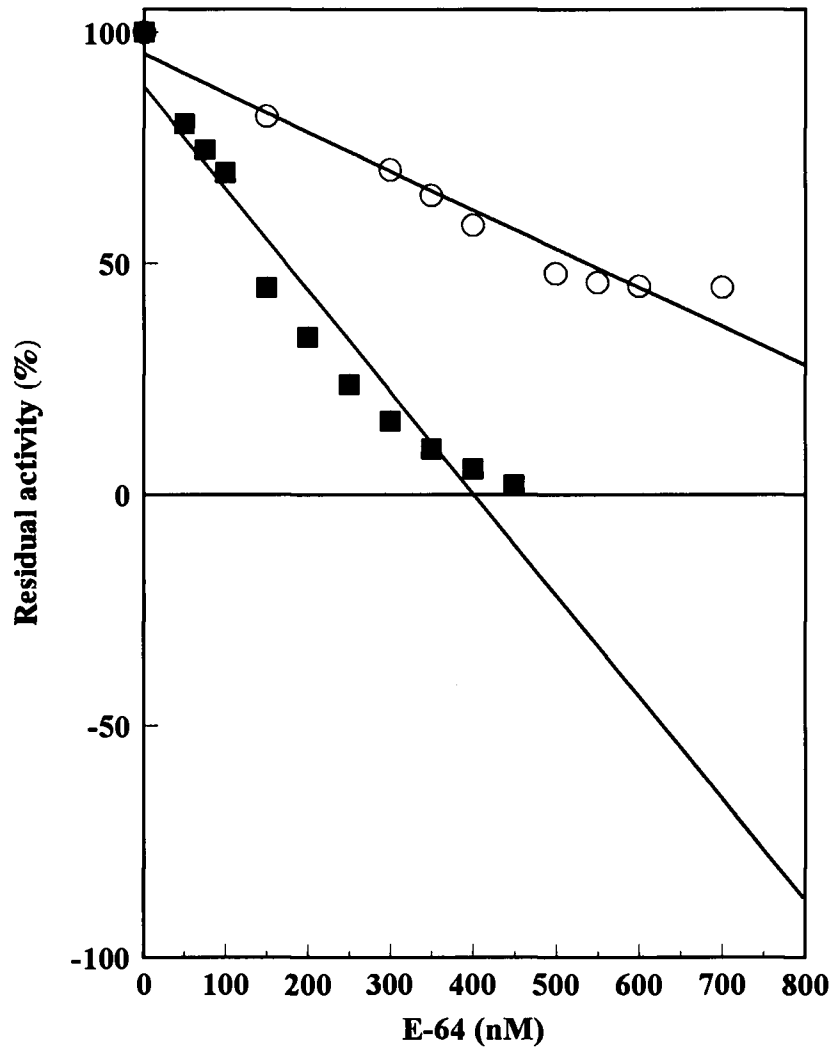

Fig. 7. Active site titration of Pacific whiting non-acidified (-○-) and acidified P-I (-■-) with E-64

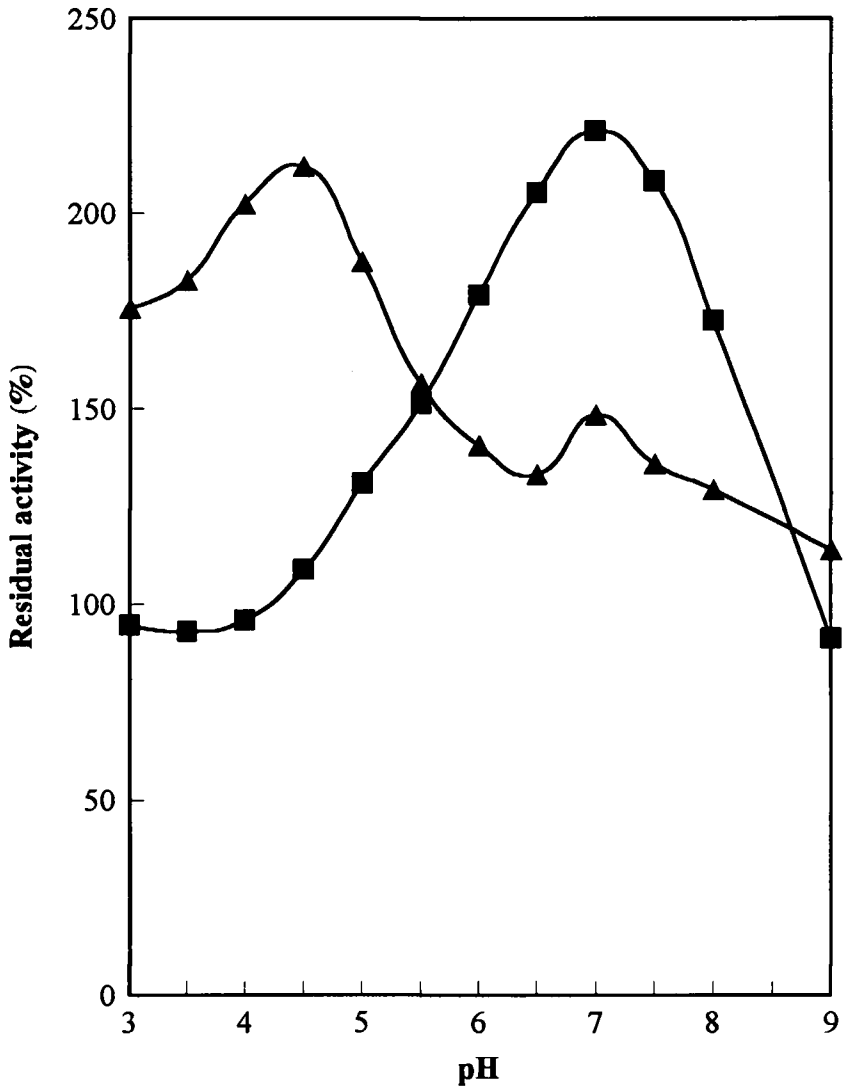

Fig 8. pH stability of Pacific whiting non-acidified (-▲-) and acidified P-I (-■-) after incubation for 30 min at 0°C

CONCLUSION

Two proteases, purified from Pacific whiting, showed similar substrate specificity and substrate affinity with Z-Phe-Arg-NMec. P-I and P-II also showed similar pH stability and preactivation profile at 30°C. Both proteases showed the same optimum temperature, 55°C. Both proteases also showed similar first order thermal inactivation profile and activation energy for heat denaturation at pH 7.0. According to kinetic properties studied, P-I and P-II were suggested to be the same enzyme. Also both enzymes were implicated as cathepsin L based on substrate specificity and pH and temperature activity profile. Both enzymes may contribute to textural degradation in cooked Pacific whiting, according to the pH optimum (pH 5.5) and the temperature optimum (55°C) of the activity. Based on the high inactivation rate of Pacific whiting P-I and P-II above 75°C, rapid cooking can be an effective method for inactivation of the proteases and controlling tissue softening.

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APPENDIX

INHIBITORY EFFECT OF FOOD GRADE PROTEINS ON AUTOLYSIS OF PACIFIC WHITING (*Merluccius productus*)

ABSTRACT

Beef plasma protein (BPP), egg white and potato extract were tested for their ability to inhibit proteolysis in fish mince and surimi made from Pacific whiting (*Merluccius productus*). There was strong inhibition by all three compounds in fish mince when measured by autolysis. However, there were significant differences among these food grade protease inhibitors when tested in surimi by autolysis. BPP showed the strongest inhibition of the proteolytic effect followed by egg white and potato extract at all the levels studied. Based on inhibition studies by various types of chemical inhibitors, it was shown that the major enzyme contributing to tissue softening of fish muscle was a cysteine protease.

INTRODUCTION

Commercial utilization of Pacific whiting (*Merluccius productus*) as surimi-based products in the U.S. has been limited by *the modori* phenomenon (thermal gel degradation) due to heat stable proteolytic enzymes in the flesh (Chang-Lee et al., 1989; Patashnik et al., 1982). High extensive tissue proteolysis occurs in minced flesh accompanied by mushiness. A considerable portion of proteolytic activity still remains in the flesh after processing into surimi through a water washing process. Partial hydrolysis of surimi proteins due to proteases in surimi hinders the formation of a gel matrix. The myofibrillar proteins were believed to be the major target for endogenous heat stable proteinases, as well as the major component involving gel formation during heat setting of surimi based products (Ouali, 1990; Toyohara and Shimizu, 1988).

Multicatalytic and serine alkaline proteases are usually responsible for gel-weakening during heat setting made from Carp (Iwata et al., 1973), White croaker (Makinodan et al., 1987; Yanagihara et al., 1991), Atlantic menhaden (Boye and Lanier, 1988), Threadfin-bream (Kinoshita et al., 1991) and Oval-filefish (Toyohara et al., 1990). Another type of heat stable proteases, acid cysteine proteases, are also involved in softening of fish muscle such as Arrowtooth flounder (*Atheresthes stomias*) (Greene and Babbitt, 1990), Swordfish (*Xiphias gladius*) (Konagaya, 1983) and Pacific hake (*Merluccius productus*) (Masaki et al., 1993). Generally, muscle degradation of this type is the result of elevated proteolytic activity during the heat process with the presence of parasites (Konagaya, 1980).

The proteolytic activity, observed in the flesh of Pacific whiting parasitized with Myxosporidian spores, was optimal at pH 5.5 when analyzed at 37°C (Konagaya and Aoki, 1981) and 5.25 when assayed at 55°C (Porter, 1992). According to the inhibitor studies, the parasitized Pacific whiting enzymes were inhibited by iodoacetic acid and *p*-chloromercuribenzoate, implying cysteine proteinase characteristics (Konagaya and Aoki, 1981). It was also shown that the compounds involved in sulfhydryl interchange with Pacific whiting proteases were the most active inhibitors (Miller and Spinelli, 1982).

Functional quality of surimi can be improved by beef plasma hydrolysate and egg white, particularly when precooked at 60°C where proteolytic enzymes can cause a weakening in gel structure (Hamann et al., 1990). Nagahisa et al. (1981) proved that dried egg white powder, oxyacidic salts, peroxides and water extracts of potato have an inhibitory effect on the proteases in Pacific whiting muscle tissue. Chang-Lee et al. (1990) reported the superiority of 3% egg white for improving the functional characteristics of Pacific whiting surimi as compared with equal levels of whey protein concentrate and soy protein isolate.

The objective of this study was 1) to test the effect of beef plasma protein, egg white, and potato extract as protease inhibitors on Pacific whiting unwashed mince and surimi; 2) to classify the proteases in Pacific whiting fish juice by various types of chemical inhibitors.

MATERIALS AND METHODS

Preparation of Surimi

Pacific whiting (*Merluccius productus*) were harvested off the Oregon coast and were processed into fillets at Astoria Seafood Sales, Inc. (Astoria, OR.) within 24 hr of capture. These fillets were kept in ice and transported to the Oregon State University Seafood Laboratory. Some fillets were processed into surimi by the method described by Chang-Lee et al. (1990). Every 600 g of surimi samples was vacuum packaged in semi-rigid containers, then kept frozen at -20°C for autolysis studies. The other fillets were kept frozen at -20°C for unwashed fish mince autolysis or fish juice preparation. Unwashed fish mince was prepared using a food grinder, model FG-A (KitchenAid, Inc. St. Joseph, MO.).

Preparation of Fish Juice

Sarcoplasmic fluid of fish muscle was prepared as a source of protease from fillets of Pacific whiting infected with myxosporidian by the method of Erickson et al. (1983). Upon thawing, the fillets were finely commuted. The chopped sample was then centrifuged at 5,000 xg (7,000 rpm, GS-3, Sorvall) for 30 min at 4°C. Muscle supernatants collected were referred to as "fish juice".

Autolysis Test on Fish mince and Surimi

Inhibitory effects on Pacific whiting autolytic activity of unwashed fish mince or surimi were investigated by various types of protein additives and proteins including beef plasma proteins (BPP) (American Meat Protein Corp.), egg white powder (Milton G. Waldbaum Corp.), potato extract (Nonpareil Corp.), and casein (Sigma Chem. Co., St. Louis, MO).

These inhibitor studies were measured according to the method of Greene and Babbitt (1990). Food grade protein additives and casein were added at levels of 0 to 4% (w/w) into 3 g of unwashed fish mince or surimi. The mixture was then incubated in a water bath at 55°C for 1 hr. Autolysis was stopped by adding 27 ml of 5% cold trichloroacetic acid solution (TCA) (J.T. Baker Inc., Phillipsburg, NJ.) to the mixture. The mixture was homogenized, incubated at 4°C for 15 min, then centrifuged at 6,100 xg for 15 min. TCA-soluble supernatant was analyzed for oligopeptide contents by Lowry's assay (Lowry et al., 1951) and expressed as nmole of tyrosine released. The inhibitory effect was expressed as percent inhibition which was calculated as follows (PI refers to protease inhibitors):

$$\% \text{ inhibition} = \frac{(\text{Tyrosine without PI} - \text{Tyrosine with PI})}{\text{Tyrosine without PI}} \times 100$$

Inhibition of the Activity by Chemicals

All four types of inhibitors (serine, cysteine, aspartic, and metallo proteases) (Asghar and Bhatti, 1987) were used to classify the protease in Pacific whiting fish juice. These chemical inhibitors included *p*-chloromercuribenzoate, EDTA, E-64 (trans-epoxysuccinyl-L-leucylamino(4-guanidono)butane), pepstatin (Calbiochem, La Jolla, CA), soybean trypsin inhibitor, dithiothreitol and phenanthroline. All chemical inhibitors, not specified previously, were purchased from Sigma Chem. Co. (St. Louis, MO). Concentration of chemical inhibitors used in this research are listed in Table 3. All activity was measured using azocasein by the method of An et al. (1994). The reaction mixture that contains 2 mg of azocasein and 625 μL of McIlvaine buffer (pH 3.5 to 9) in total volume of 1.0 mL was prequilibrated at 55°C. Then 250 μL of fish juice, expressed as protease content, was added into the reaction mixture and incubated at 55°C for 1 hr. Proteolytic reaction was stopped by adding 200 μL of cold 50% (W/V) trichloroacetic acid (TCA) solution and the mixture was kept at 4°C for 15 min. After centrifuging at 5,700 xg for 10 min (Eppendorf Micro Centrifuge, Model 5415C, Brinkmann, NY), 800 μL of TCA-soluble supernatant pretreated by 60 μL of 10 N NaOH to enhance intensity of the azo color was used for absorbance assay. Absorbance of the reaction mixture was measured spectrophotometrically at the wavelength of 450 nm. (Spectrophotometer, DU640, Beckman Instruments, Inc., Salt Lake City, UT). Inhibitory effect was expressed as percent inhibition which was calculated as following (PI refers to protease inhibitors).

$$\% \text{ inhibition} = \frac{(A_{450}\ \text{without PI} - A_{450}\ \text{with PI})}{A_{450}\ \text{without PI}} \times 100$$

RESULTS AND DISCUSSION

Inhibitory Effect of Food Grade Proteins on Autolysis of Fish Mince

All food grade protein additives were effective in inhibiting protease activity in the fish mince assay (Fig. 10). At 1% level, there was a 70% and 76% inhibition in unwashed fish mince by potato extract and BPP, while 55% inhibition was found for egg white. At a higher level, there was similar inhibitory effect among the protein additives.

Generally, potato extract showed a good inhibitory effect against Pacific whiting fish mince. The level of inhibition was similar to BPP, but it was less effective at 1%. Egg white was not as effective as BPP or potato extract in inhibiting autolysis of the fish mince. Its inhibitory effect at 1 or 2% level was lower than that of BPP or potato extract. Nevertheless, the effect was comparable at higher levels of 3 and 4%.

Casein was added as a negative control to determine whether competitive inhibition, via increased protein content, was a factor. Less than 10% inhibition was observed from 0 to 4% level studies for casein.

Inhibitory Effect of Food Grade Proteins on Autolysis of Surimi

Among the protein additives tested, BPP showed the highest inhibitory effect on Pacific whiting surimi proteases up to 4% (Fig. 11). The inhibitory effect of egg white on surimi proteases was not as effective as BPP at 1% level. However, it was as effective as BPP at higher levels. Potato extract showed the lowest inhibitory effect at all levels tested.

Protease inhibition in fish mince and surimi showed different results when measured by autolysis, particularly for egg white and potato extract. It is possible that the target proteses of these inhibitors might be different for fish mince and surimi.

Inhibitory Effect of chemicals on Fish Juice

All four types of inhibitors (serine, cysteine, aspartic, and metallo proteases) were tested with Pacific whiting fish juice to determine the class of proteases regarding its active site. Among the tested inhibitors, the apparent inhibitions (almost 100%) were shown by E-64 at each pH studies (Fig. 12). It can be suggested that the major proteases responsible for thermal degradation of Pacific whiting surimi belong to cysteine proteases.

Table 4. Concentration of chemical protease inhibitors used for characterization of enzymes in Pacific whiting fish juice.

Protease Inhibitors	Concentration
E-64	10 μM
1,10-Phenanthroline	1 mM
Dithiothreitol + EDTA	2 mM each
p-Chloromercuribenzoate	1 mg/L
Soy Trypsin Inhibitor	100 mg/L
Pepstatin	1 mg/L

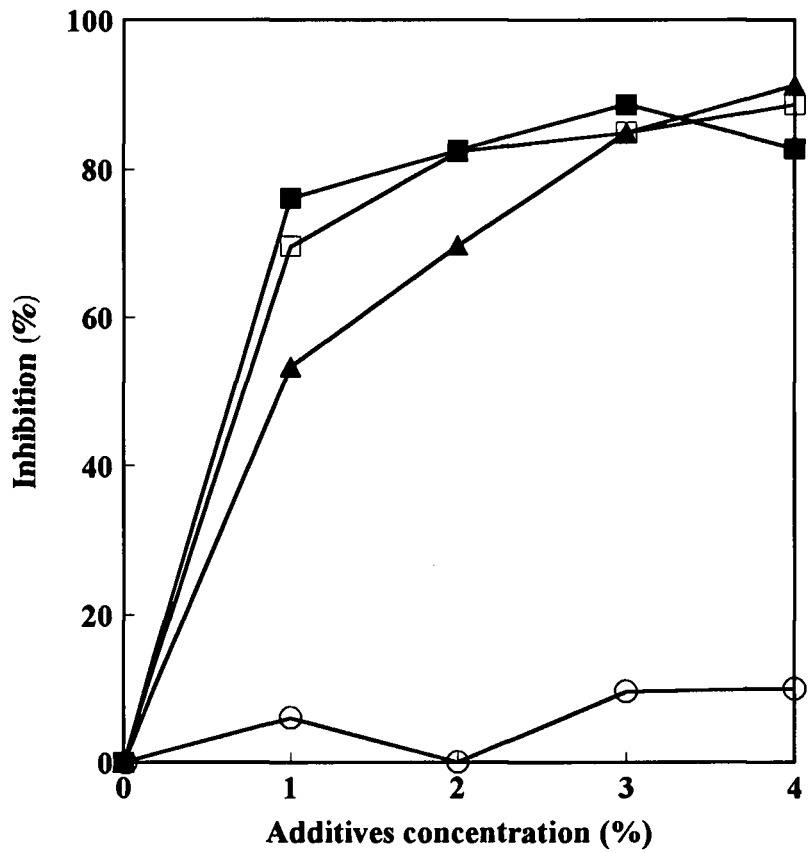

Fig. 9. Effect of food grade protein additives, BPP (-■-), egg white (-▲-), potato extract (-□-), and casein (-○-) on autolysis of Pacific whiting fish mince for 1 hr at 55°C

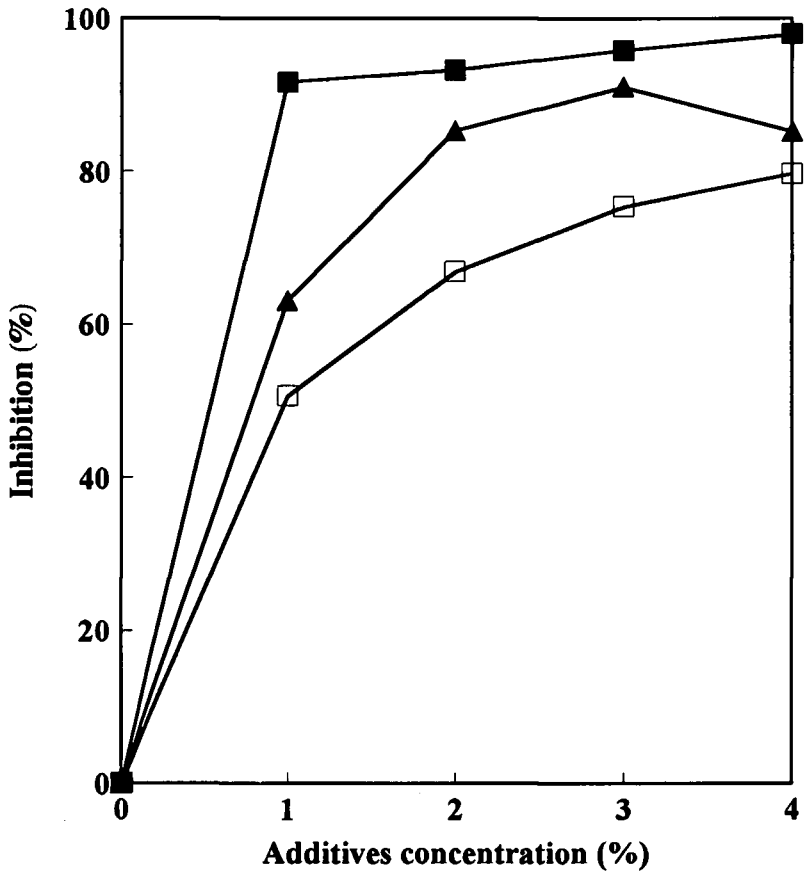

Fig. 10. Effect of food grade protein additives, BPP (-■-), egg white (-▲-), and potato extract (-□-) on autolysis of Pacific whiting surimi for 1 hr at 55°C

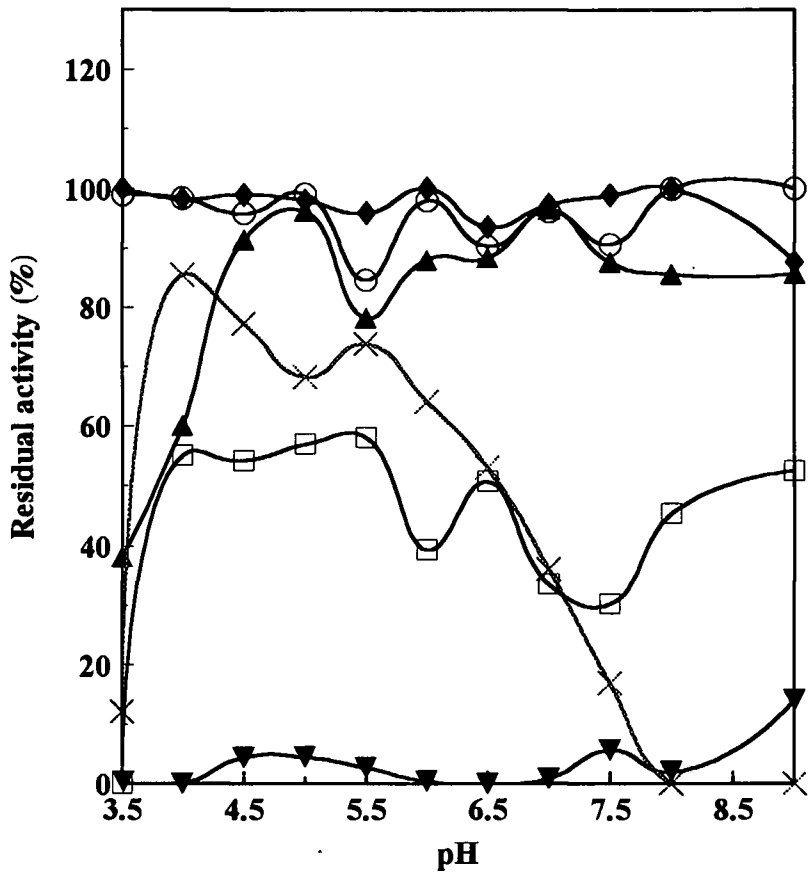

Fig. 11. Effect of chemical protease inhibitors, 1,10-phenanthroline (□-□), dithiothreitol and EDTA(-×-), *p*-chloromercuribenzoate (-▲-), pepstatin (-◆-), soybean trypsin inhibitor (-○-), E-64 (-▼-) on Pacific whiting fish juice at various pH

CONCLUSIONS

Based on autolysis on fish mince and surimi, BPP is the most effective inhibitor among the food grade protein additives assayed. Potato extract shows less effective than BPP at 1% level, but similar inhibitory effect at a higher concentration study in the autolysis of fish mince. Egg white showed less inhibitory effect than potato extract and BPP at 1% level in the autolysis of fish mince but similar at higher concentration level. However, it displayed better inhibitory effect than potato extract at 1 and 2% level when measured in the autolysis of surimi. According to chemical inhibitors assay, it is suggested that crude proteases in fish juice have apparent characteristics of cysteine protease by E-64.

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