Characterization of Active Components in Food-Grade Proteinase Inhibitors for Surimi Manufacture

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Commonly used food-grade inhibitors, beef plasma protein (BPP), egg white, and potato powder, were characterized for their inhibitory activity toward proteinases. BPP and egg white had the highest rate of cysteine and serine proteinase inhibition, respectively. Egg white, containing specific serine proteinase inhibitors, reduced trypsin activity by as much as 99%. Active inhibitory components in the proteinase inhibitors were detected by inhibitory activity staining on sodium dodecyl sulfate (SDS)-substrate gels. Egg white and potato powder contained more serine proteinase inhibitor bands than cysteine proteinase inhibitor bands. No specific cysteine proteinase inhibitory component was found in BPP. Serum albumin was detected on both papain and trypsin inhibitory activity-stained gels of BPP but was not inhibitory to the proteinases. A high molecular weight protein band (HMP) of BPP was also detected on both inhibitory activity-stained gels, but the protein was postulated to be polymerized plasma components resistant to proteolysis.

Keywords: *Activity staining; proteinase inhibitor; surimi*

INTRODUCTION

Pacific whiting (*Merluccius productus*) is the most abundant fishery resource off the U.S. Northwest coast with a harvest of 180 000 metric tons in 1994 (Talley, 1995). The majority of the Pacific whiting harvest is used to produce surimi, the main ingredient for seafood analogs.

Pacific whiting is difficult to use, since it undergoes proteolytic degradation of its myofibrillar proteins. The degradation interferes with gel formation and, consequently, causes gel strength loss in surimi. Myofibrillar degradation was highest at 55 °C (Chang-Lee et al., 1989; Morrissey et al., 1993), and crude proteinase preparations showed maximal activity at 55 °C and pH 5.5 (An et al., 1994; Chang-Lee et al., 1989). A proteinase was purified from Pacific whiting fish fillets and identified as cathepsin L (Masaki et al., 1993; Seymour et al., 1994). Properties of the purified cathepsin L from Pacific whiting reflected the autolytic activity in surimi (An et al., 1994; Seymour et al., 1994). Cathepsin L is an active lysosomal cysteine proteinase of various protein substrates (Kirschke et al., 1977, 1982; Koga et al., 1990; Yamashita and Konagaya, 1991).

Several proteinase inhibitors have been used to improve the physical properties of surimi gels (Morrissey et al., 1993; Reppond and Babbitt, 1993; Wasson et al., 1992a). The most commonly used food grade inhibitors are beef plasma protein (BPP), egg white, and potato powder. These additives show various degrees of proteinase inhibition (Akazawa et al., 1993; Morrissey et al., 1993; Porter et al., 1993; Reppond and Babbitt, 1993). BPP and egg white are effective in controlling autolysis of Atlantic menhaden surimi or mince (Hamann et al., 1990), Alaska pollock (Hamann et al., 1990; Reppond and Babbitt, 1993), arrowtooth flounder (Reppond and Babbitt, 1993; Wasson et al., 1992a) and Pacific whiting (Akazawa et al., 1993; Morrissey et al., 1993). Potato powder containing high amounts of

proteinase inhibitors (Porter et al., 1993) slowed autolysis of Pacific whiting (Akazawa et al., 1993; Morrissey et al., 1993), arrowtooth flounder (Porter et al., 1993), and trout muscle (Kaiser and Belitz, 1973).

Even though food-grade inhibitors have been successfully used to improve the gel strength of surimi by controlling proteolytic activities during surimi production, questions remain concerning the identity of the inhibitory components and the mechanisms of inhibition. The objective of this study was to characterize and identify inhibitory components in the most commonly used food-grade inhibitors for surimi.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA, fraction V, 98–99% albumin), ovoinhibitor, ovomucoid, papain (double crystallized), α-*N*-benzoyl-DL-arginine-2-naphthylamide (BANA), benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), β-naphthylamide, Brij 35, cysteine, phenylmethanesulfonyl fluoride (PMSF), Triton X-100, bovine pancreas trypsin, and L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane (E-64), were purchased from Sigma Chemical Co. (St. Louis, MO). Fast Garnet GBC base (4-amino-2,3-dimethylazobenzene) was obtained from Aldrich Chemical Co. (Milwaukee, WI), and α₂-macroglobulin (α₂M) from Calbiochem Corp. (La Jolla, CA). A low molecular weight calibration kit was purchased from Pharmacia Biotech, Inc. (Piscataway, NJ), and high molecular weight protein standards were purchased from Sigma Chemical Co. Casein (sodium caseinate) and mersalyl acid {*o*-[3(hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetic acid} were purchased from U.S. Biochemical Corp. (Cleveland, OH), and sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA). Beef plasma protein (BPP) (AMP 600N) was obtained from the American Meat Protein Corp., Inc. (Ames, IA), egg white (standard spray dried egg albumin) was obtained from the Monark Egg Corp. (Kansas City, MO), and potato powder (GS-91) was obtained from the Nonpareil Corp. (Black Foot, ID).

Preparation of Inhibitor Stock Solutions. Stocks of BPP, egg white, BSA, and casein were prepared each day at 4% (w/v) for enzyme inhibition assays. Stock was diluted with water to prepare 1%, 2%, and 3% solutions. For egg white, the stock solution was homogenized at low speed with a Polytron (Brinkmann Instruments, Westbury, NY). For potato powder, the 1, 2, 3, and 4% solutions were prepared individually from the powder.

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Determination of Protein Content of Inhibitor Solutions. Protein content was determined by extracting the total proteins from 1% inhibitor solutions by the method of An et al. (1994). For potato powder, 10% solutions were used due to the low protein content. Proteins were solubilized in 5% (w/v) SDS at 80 °C for 1 h. All of the inhibitors, except potato powder which had a high starch content, were completely solubilized. The insoluble residues were precipitated by centrifugation (Sorvall Model RC-5B, Rotor SS-34, DuPont Co., Newtown, CT) at 7000g for 15 min at room temperature. The protein content of the supernatant was determined by the Lowry assay (Lowry et al., 1951) using BSA as the standard.

Papain Activity Inhibition Assay. Cysteine proteinase inhibitory activity was measured with papain, as a model, by the method of Izquierdo-Pulido et al. (1994) using BANA as a synthetic substrate (Barrett, 1972). Solutions containing 50 μL of papain (0.045 mg/mL) and 50 μL of 1−4% inhibitor solutions were added to 100 mM phosphate buffer (pH 6.0) containing 1.33 mM EDTA and 2.7 mM cysteine to a final volume of 400 μL. The mixture was preincubated for 5 min at 40 °C, and 10 μL of 40 mg/mL BANA in dimethyl sulfoxide was added to start the reaction. The reaction mixture was incubated at 40 °C for precisely 10 min. A 400 μL aliquot of a color reagent containing 0.1 mM Fast Garnet GBC base, 0.2 mM sodium nitrite, 2% Brij 35, and 5 mM mersalyl acid was added to terminate the reaction. After allowing 10 min for color development, the solution was centrifuged at 8000g for 3 min (Eppendorf Micro Centrifuge, Model 5415C, Brinkmann, New York, NY). Papain activity was measured as absorbance at 520 nm (Beckman Instruments, Inc., Redmond, WA) due to formation of β-naphthylamide. Standards and blanks were prepared by replacing the enzyme with 50 μL of 20 mM β-naphthylamide and water, respectively. For each assay, a positive and a negative control were run by adding 0.1 mM E-64 and the buffer, respectively, instead of inhibitor.

Trypsin Activity Inhibition Assay. Serine proteinase inhibitory activity was measured with trypsin, as a model, by the method of Smith et al. (1980) using BAPNA as a synthetic substrate. Solutions containing 200 μL of 20 μg/mL trypsin, 100 μL of 1−4% inhibitor solutions, and 100 μL of water were preincubated for 10 min at 37 °C. The reaction was initiated by adding 500 μL of BAPNA (0.4 mg/mL) to the mixture. After incubating at 37 °C for 10 min, 100 μL of 30% (v/v) acetic acid was added to terminate the reaction. The mixture was centrifuged at 8000g for 3 min (Eppendorf Micro Centrifuge). The residual trypsin activity was measured spectrophotometrically at 410 nm (Beckman Instruments, Inc.) due to p-nitroaniline. For each assay, a positive and a negative control were run by adding PMSF and water, respectively, instead of the inhibitors.

SDS-Substrate Gel and Staining for Inhibitory Components. Inhibitory proteins in the inhibitor compounds were analyzed on 15% SDS-substrate gels, unless otherwise specified, by the method of García-Carreño et al. (1993) with slight modifications. Proteinase inhibitor solutions were mixed 1:1 (v/v) with sample treatment buffers containing no β-mercaptoethanol (BME). Fifteen micrograms of proteins was applied onto the gel without prior boiling. The proteins were electrophoresed on a Mini-Protean II unit (Bio-Rad Laboratories) at a constant voltage of 150 mV for 90 min on ice.

Two identical gels were prepared: one gel was fixed and stained for total proteins with Coomassie Blue R-250 as a control and the other was stained for inhibitory activity as follows. The electrophoresed gel was washed in 2.5% Triton X-100 for 15 min to remove SDS and renature the proteins (Izquierdo-Pulido et al., 1994). The gels were washed with distilled water and incubated with 0.2 mg/mL papain in 0.1 M phosphate, pH 6.0, containing 1 mM EDTA and 2 mM cysteine or 0.2 mg/mL trypsin in 0.05 M Tris-HCl, pH 8.2, containing 20 mM CaCl$_2$. After allowing the enzyme to diffuse into the gel at 0−5 °C for 30 min, the gels were washed with distilled water and incubated in 1% (v/v) casein solution in the relevant buffers (0.1 M phosphate, pH 6.0, for papain and 0.05 M Tris-HCl, pH 8.2, for trypsin) for 90 min at 37 °C. The gels were fixed and stained with Coomassie Blue R-250.

Table 1. Protein Contents of 1% (w/v) Proteinase Inhibitor Solutions

inhibitors	protein concn[a] (mg/mL)
BPP	12.6
egg white	15.7
potato powder	1.1

[a] Average of two replicates measured with BSA as a standard.

Nondigested stained bands were tentatively identified as inhibitory proteins. Molecular weight was determined by comparing with that of control gel, since molecular weight standards on activity-stained gel were completely hydrolyzed. The apparent molecular weight of the inhibitory components was calculated (Weber and Osborn, 1969) using the low molecular weight protein standard [phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactalbumin (14 400)].

For identification of α_2M in BPP, 12% gels were used for better resolution, and the electrophoresis running time was doubled to improve separation of proteins in the high molecular weight region. The high molecular weight protein standard [myosin (205 000), β-galactosidase (116 000), phosphorylase b (97 400), bovine albumin (66 000), egg albumin (45 000), and carbonic anhydrase (29 000)] was used as a reference.

Activity Assay of Proteinases on Protein Substrates. Activities of papain and trypsin were analyzed with casein or azocasein according to the method of An et al. (1994). A reaction mixture containing 2 mg of casein or azocasein, 0.625 mL of relevant buffer (0.1 M phosphate, pH 6.0, containing 1 mM EDTA and 2 mM cysteine for papain; and 0.05 M Tris-HCl, pH 8.2, containing 20 mM CaCl$_2$ for trypsin), and water was preincubated at 37 °C for 5 min. The enzyme was then added, and its activity was measured at 37 °C for 5 min. The total volume of the reaction mixture, including enzyme, was maintained at 1.25 mL. The reaction was terminated by adding 0.2 mL of cold (4 °C) 50% (w/v) trichloroacetic acid (TCA). After allowing unhydrolyzed proteins to precipitate at 4 °C for 5 min, samples were centrifuged at 6000g for 5 min (Eppendorf Micro Centrifuge). For azocasein, TCA-soluble protein recovered from the supernatant was mixed with 10 N NaOH (800:60 v/v), and the absorbance of the azo compound at 428 nm was determined. For casein, TCA-soluble proteins were recovered in the supernatant, and its equivalent tyrosine content was determined by the Lowry assay (Lowry et al., 1951). Enzyme activity was expressed either as ΔA_{750} or U/mL. One unit of activity was defined as releasing 1 nmol of tyrosine/min.

RESULTS

Protein Content of Inhibitors. All food-grade inhibitor compounds were highly proteinaceous except potato powder (Table 1). Protein content of the 1% solutions prepared from BPP and egg white was 12.6 and 15.7 mg/mL, respectively. The protein content was higher than 10 mg/mL for 1% inhibitor solutions due to different amino acid profiles from BSA used as a standard. Protein content of 1% potato powder solution was about 1.1 mg/mL with the rest likely starch (Porter et al., 1990), indicating approximately 10-fold lower protein content than those of other inhibitor compounds.

Optimization of Enzyme Concentration for Papain and Trypsin Inhibition Assays. Optimum enzyme concentrations were determined for papain and trypsin inhibition assays using synthetic substrates with BPP. The optimum enzyme concentrations were found to be 0.045 mg/mL for papain (Figure 1) and 0.020 mg/mL for trypsin (Figure 2). At these enzyme concentrations, the assays showed the largest activity differences with and without the added inhibitor, 4% BPP. At higher enzyme concentrations, the inhibitor did not
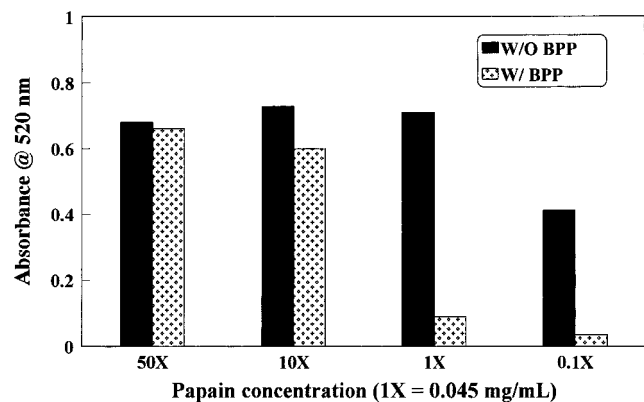
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Figure 1. Effect of 4% BPP on papain activity at various concentrations. Papain was preincubated with or without 4% BPP prior to measuring activity with BANA.
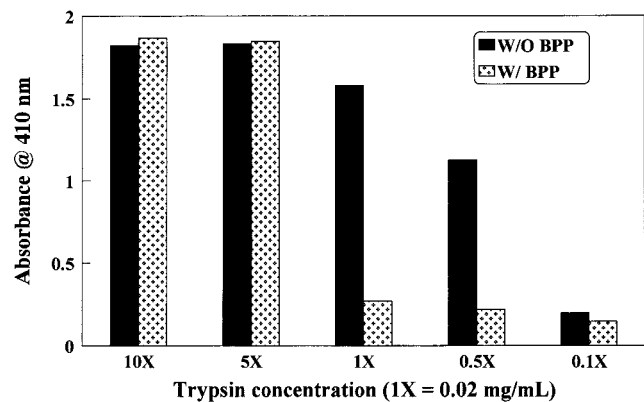

Figure 2. Effect of 4% BPP on trypsin activity at various concentrations. Trypsin was preincubated with or without 4% BPP prior to measuring activity with BAPNA.
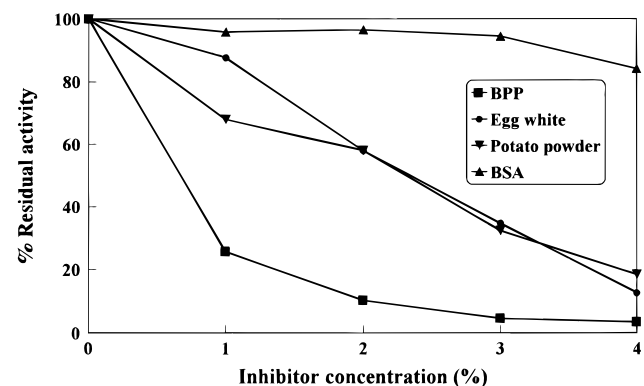

Figure 3. Inhibition of papain activity by various proteinase inhibitors with BANA as the substrate.

Figure 4. Inhibition of trypsin activity by various proteinase inhibitors with BAPNA as the substrate.

Figure 5. SDS-PAGE patterns of food-grade proteinase inhibitors. B, beef plasma protein; E, egg white; P, potato powder; S, bovine serum albumin; C, casein; and M, molecular weight standards. Proteins, 15 μg, were applied on a 15% gel.

lower the enzyme activity, indicating insufficient inhibitor was present. At lower enzyme concentrations, the activity was too low to measure accurately.

Estimation of Cysteine Proteinase Inhibitors. BPP had the highest content of cysteine proteinase inhibitors among the additives studied (Figure 3). At 1% BPP, residual activity was 26% of control, which decreased gradually and reached a plateau at 3%. This indicated that increasing the BPP level above 3% would not enhance the efficiency of inhibition. For egg white, a dose-related response was found between the inhibition and the inhibitor concentration. Papain activity decreased proportionally with increased egg white, reaching 13% residual activity with 4% egg white. The inhibitory efficiency of potato powder was similar to that of egg white, although inhibition at 1% potato powder
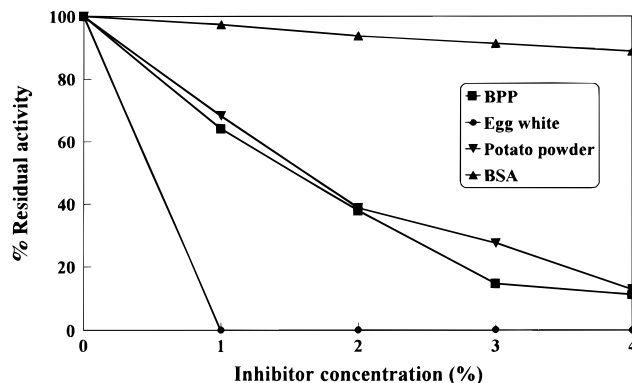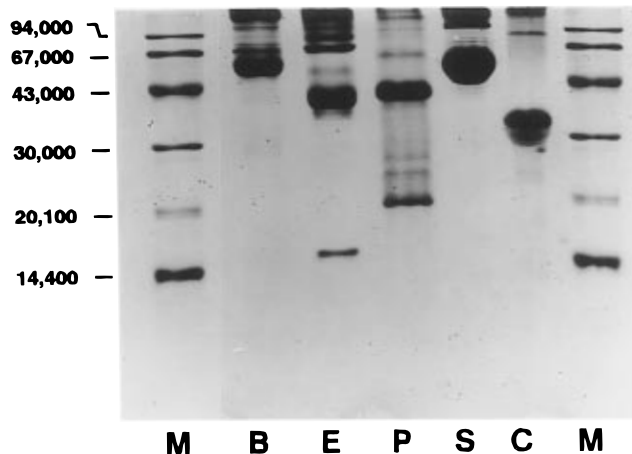
(68%) was higher than that for egg white (88%). BSA, used as the negative control, did not affect the residual activity up to 3%, although a slight decrease was seen at 4%.

Estimation of Serine Proteinase Inhibitors. Trypsin was completely inhibited by 1% egg white due to the presence of the highly specific serine proteinase inhibitors (Figure 4), ovomucoid, a specific trypsin inhibitor, and ovoinhibitor, a general serine proteinase inhibitor which can inhibit both trypsin and chymotrypsin (Kassell, 1970; Osuga and Feeney, 1977). Egg white consists of 11% ovomucoid and 1.5% ovoinhibitor by weight (Osuga and Feeney, 1977). With 1% BPP or potato powder, similar residual activities of 64 and 68%, respectively, were found. The inhibition of trypsin by BPP and potato powder was proportional to inhibitor concentration.

Identification of Inhibitory Components. All the proteins of the inhibitors were shown in a control gel (Figure 5), and inhibitory components were identified as stained bands remaining after treatment with cysteine and serine proteinases, respectively (Figures 6 and 7).

BPP. Two major components were found in BPP, including BSA and a currently unidentified high molecular weight protein (HMP) (Figure 5). The two bands were also detected on inhibitor activity-stained gels for both papain and trypsin (Figures 6 and 7). The intensity of the HMP band and BSA was reduced on both the papain and trypsin inhibitor activity-stained gels, compared with the control. However, BSA was not
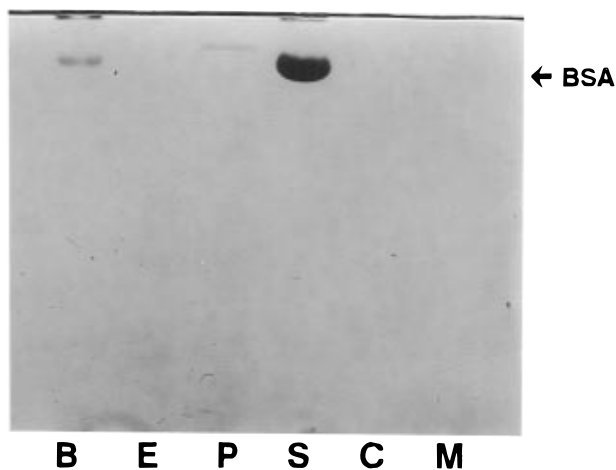
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Figure 6. Food-grade proteinase inhibitors stained for papain inhibitory activity at 37 °C. B, beef plasma protein; E, egg white; P, potato powder; S, bovine serum albumin; C, casein; and M, molecular weight standards. Proteins, 15 μg, were applied on a 15% gel.
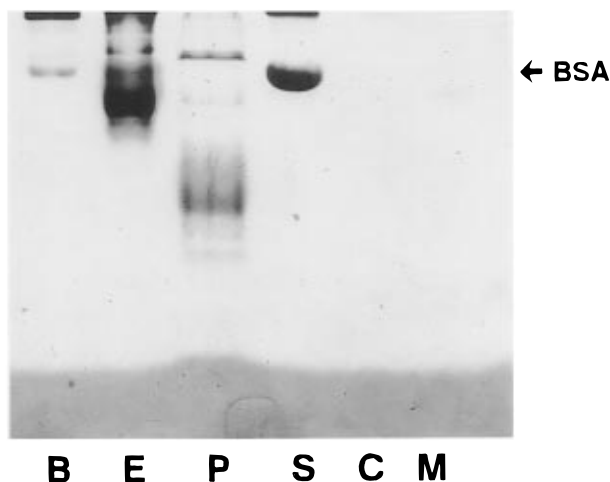

Figure 7. Food-grade proteinase inhibitors stained for trypsin inhibitory activity at 37 °C. B, beef plasma protein; E, egg white; P, potato powder; S, bovine serum albumin; C, casein; and M, molecular weight standards. Proteins, 15 μg, were applied on a 15% gel.

inhibitory to either papain or trypsin as discussed below in the section Effect of BSA on Proteinases. A few minor bands were also present between the BSA and HMP bands (Figure 5), but they did not show any inhibitory activity toward either papain or trypsin.

Inhibitory activity of α_2M, implicated as the major proteinase inhibitor in BPP (Hamann et al., 1990), was tested by inhibitory activity staining (Figure 8). α_2M is a large molecular weight (M_r 718 000) plasma protein consisting of approximately 3% beef plasma proteins (White et al., 1973) and inhibits proteolytic enzymes from all four catalytic classes (Sottrup-Jensen et al., 1990; Starkey and Barrett, 1977). Purified α_2M after inhibitory staining was present with both trypsin and papain treatment, although less remained after papain treatment (Figure 8). However, the α_2M band on the inhibitory activity-stained gel of BPP was obscured due to a diffused band, assumed to be the HMP band on the 15% SDS-substrate gel (Figure 5). Previously, it was reported that gel strength of arrowtooth flounder surimi was increased by adding α_2M with no evidence of myosin degradation on electrophoresis for surimi treated with α_2M (Wasson et al., 1992a). Hoki surimi treated with

α_2M had better gel stress and strain values compared to untreated control (Lorier and Aitken, 1991).

The HMP band was highly resistant to proteolysis and appeared on both papain and trypsin inhibitory activity-stained gels. This band might be proteins polymerized by the clot-forming proteins in plasma (White et al., 1973). The polymerized proteins by covalent bonds are resistant to proteolysis (Folk, 1980).

Egg White. Egg white contained several trypsin inhibitors as shown by the trypsin inhibitor-stained gel (Figure 7). The most active trypsin inhibitor appeared as a wide band whose midpoint suggested an apparent molecular weight of 40 900. This band was identified as ovoinhibitor using the purified protein as a reference (Weerasinghe and An, 1995). Ovoinhibitor is reported to have M_r 49 000 (Stevens, 1991). The band appeared darker after inhibitor activity staining than on the control gel, indicating effective inhibition of the proteinase. Another important inhibitory component in egg white is ovomucoid (Stevens, 1991). However, purified ovomucoid (M_r 28 000) migrated to an apparent M_r 65 590 on the nondenaturing gel condition used in this study and overlapped with ovoinhibitor band, making it unavailable to see its inhibitory effect.

A high molecular weight protein band, inhibitory to trypsin on the upper part of the gel, was postulated to be ovostatin on the basis of the molecular weight (Figure 7). Ovostatin, whose molecular weight is 780 000, is reported to be homologous to α_2M in molecular structure, function, and mechanism of inhibition (Nagase et al., 1985). The presence of cystatin (M_r 13 100) has also been reported in egg white (Stevens, 1991). However, in this study, we were unable to detect cystatin, probably due to its low concentration, 0.01% (Osuga and Feeney, 1977; Stevens, 1991). Ovalbumin, the major protein in egg white, did not show any inhibitory activity against trypsin or papain, as was also seen by Akazawa et al. (1993) in Pacific whiting surimi.

Potato Powder. One component in potato powder, whose apparent M_r was 67 870, was detected on the papain-inhibitory gel (Figure 6) as well as the trypsin-inhibitory gel (Figure 7). In addition, potato powder contained numerous serine proteinase inhibitors (Figure 7). A protein band with apparent M_r 31 290 was probably a more specific serine proteinase inhibitor, as shown by its stronger intensity on the inhibitor activity stained gel. Potato tubers contain a number of proteinase inhibitors, which include a cysteine proteinase inhibitor (Rowan et al., 1990); an aspartic proteinase inhibitor (Mares et al., 1989); inhibitor I and II for trypsin and chymotrypsin (Bryant et al., 1976; Melville and Ryan, 1972); inhibitors for pancreatic carboxypeptidase A and B, a metalloproteinase (Ryan, 1974); and an inhibitor for serine endopeptidases and metallocarboxypeptidases (Hass et al., 1976). Among those listed, the most common inhibitors in potato powder were serine or metalloproteinase inhibitors, and only one was a cysteine proteinase inhibitor. However, potato powder has been shown to be inhibitory against autolysis of Pacific whiting (Akazawa et al., 1993; Morrissey et al., 1993), trout muscle (Kaiser and Belitz, 1973), and arrowtooth flounder (Reppond and Babbitt, 1993), even though a cysteine proteinase has been reported to be the most active in Pacific whiting and arrowtooth flounder (An et al., 1994; Wasson et al., 1992b).

Effect of BSA on Proteinases. The effect of BSA on papain and trypsin activity was measured with both azocasein and casein because of the different mecha-
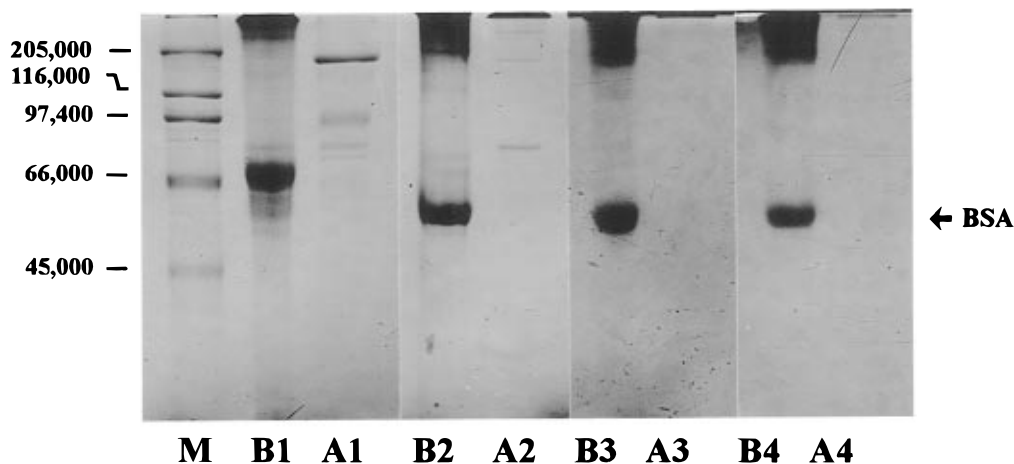
Figure 8. Purified α_2M (A) and BPP (B) on 12% SDS-substrate gel. The proteins were separated using (1) reducing condition (with BME) and stained for proteins; (2) nonreducing condition (without BME) and stained for proteins; (3) nonreducing condition and stained for inhibitory activity for papain; and (4) nonreducing condition and stained for inhibitory activity for trypsin. M, high molecular weight protein standards.
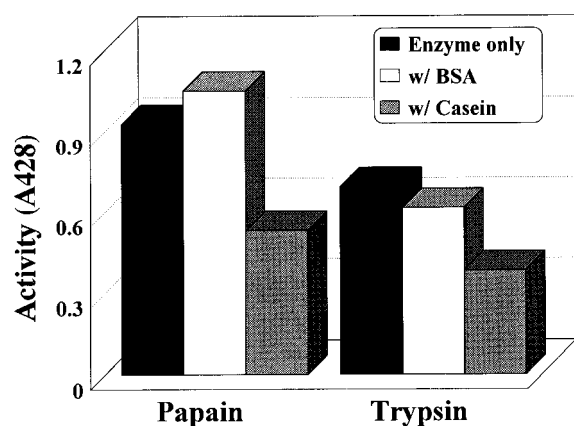

Figure 9. Effect of BSA on hydrolysis rate of papain and trypsin with azocasein. Casein was used as a reference. Enzyme activity was expressed as ΔA_{428}.
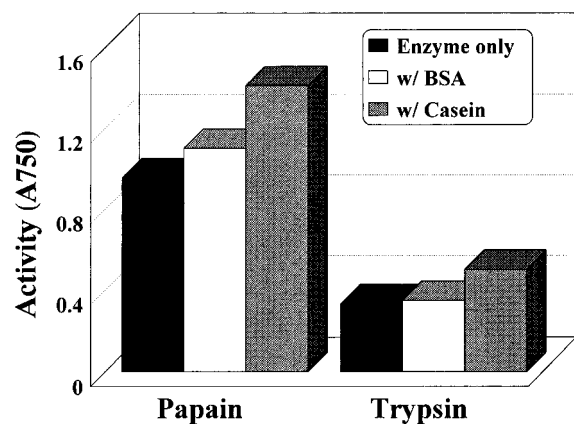

Figure 10. Effect of BSA on hydrolysis rate of papain and trypsin with casein. Casein was used as a reference. Enzyme activity was expressed as ΔA_{750}.

nisms by which they measure hydrolysis (Figures 9 and 10). The azocasein assay only reflects hydrolysis of the azo-dye-bound substrate, since azo dye in the TCA-soluble supernatant is monitored to estimate the degree of hydrolysis. In contrast, casein assay reflects the hydrolysis of all proteins in the assay mixture including substrate and inhibitors, which become TCA soluble.

A 43% and 45% decrease in hydrolyzed azocasein with papain and trypsin was observed, respectively, when the enzymes were incubated with casein (Figure 9), prima-
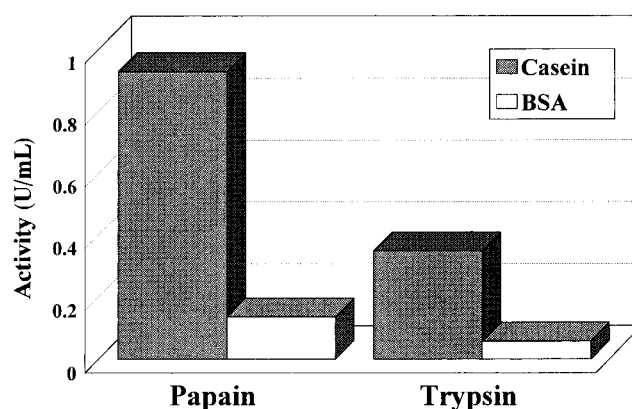

Figure 11. Substrate specificities of BSA and casein for papain and trypsin. Papain and trypsin activities were analyzed using BSA and casein at 37 °C. One unit of activity was defined as that releasing 1 nmol of tyrosine equivalent/min.

rily due to the competition between azocasein and casein. However, no significant change was detected in azocasein hydrolysis in the presence of BSA, indicating that BSA does not compete as well as casein. Incubation of papain and trypsin with casein resulted in a protein hydrolysis rate of 148% and 151%, respectively, compared to the control without casein (Figure 10). Casein, in this case, contributed to the total substrate available for enzyme hydrolysis, resulting in a higher protein hydrolysis rate. With BSA, hydrolysis by papain and trypsin increased only 15% and 5% compared to the control, indicating very little impact of BSA on enzyme hydrolysis. To confirm this result, papain and trypsin activities were measured on BSA or casein as a sole source of substrate (Figure 11). BSA showed little hydrolysis by both papain and trypsin. BSA hydrolysis was only 16% and 14% of casein for papain and trypsin, respectively. These results clarify why BSA was seen on both papain and trypsin inhibitory gels. BSA was not hydrolyzed effectively by the enzymes. Therefore, the BSA detected on the inhibitor-stained gels could not be interpreted as inhibitory.

DISCUSSION

Four catalytic classes are known for endolytic proteinases: cysteine, serine, aspartic acid, and metalloproteinases (Asghar and Bhatti, 1987). Among these four, cysteine proteinases play the most significant role

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in tissue softening and degradation of surimi proteins of Pacific whiting (An et al., 1994; Seymour et al., 1994), arrowtooth flounder (Wasson et al., 1992b), white croaker (Makinodan et al., 1987), Atlantic croaker (Lin and Lanier, 1980), and chum salmon (Yamashita and Konagaya, 1990). Therefore, inhibitor compounds should contain a higher ratio of cysteine proteinase-specific inhibitory components than other types to control autolytic activities in these fish muscles. On the basis of the cysteine proteinase inhibition result, BPP was shown to be superior to egg white or potato powder. It has been reported that the increased gel strength of Pacific whiting surimi with the addition of BPP was greater than with egg white or potato powder (Morrissey et al., 1993). Myosin was protected from proteolysis during a 60 °C heat treatment prior to rapid cooking. Despite these results, the intensity of inhibitory components detected on papain and trypsin inhibitor-stained gels was not as great as expected for the increased gel strength. On the inhibitory activity-stained gels, only two protein bands were detected as specifically inhibitory or resistant to proteolysis of cysteine proteinase activity: (1) HMP found in BPP and (2) a potato powder protein with an apparent M_r 67 870. At present, the HMP of BPP is considered cross-linked and/or polymerized proteins of plasma components, which may be highly resistant to proteolysis rather than inhibitory to proteinases.

Previous reports indicate that it is difficult to correlate gel strength of surimi solely on the inhibition of proteinase. Wasson et al. (1992b) stated that inhibitors incorporated in arrowtooth flounder surimi displayed enhanced gelling effects at levels beyond those needed to prevent proteolytic activity. Additional ingredients, such as starch in potato powder and heat-coagulable protein in egg albumin and bovine plasma, also increased gel strength (Porter et al., 1993). Therefore, it is possible that increased gel strength by these compounds might be due to a combination of effects of proteinase inhibition and gel enhancement. For example, transglutaminase, which can cross-link proteins to increase the gel strength of surimi, has been found in fish muscles as well as plasma (Hamann et al., 1990; Kishi et al., 1991; Jiang and Lee, 1992; Kim et al., 1993). Therefore, the possibility cannot be ruled out that other factors in the additives contribute to gel enhancement of surimi. Examination of the surimi additives for activities other than proteinase inhibition will be helpful in elucidating their complete function in surimi gelation.

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LITERATURE CITED

Akazawa, H.; Miyauchi, Y.; Sakurada, K.; Wasson, D. H.; Reppond, K. D. Evaluation of protease inhibitors in Pacific whiting surimi. *J. Aquat. Food Prod. Technol.* **1993**, *2* (3), 79−95.

An, H.; Seymour, T. A.; Wu, J. W.; Morrissey, M. T. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. *J. Food Sci.* **1994**, *59*, 277−281.

Asghar, A.; Bhatti, A. R. Endogenous proteolytic enzymes in skeletal muscle: their significance in muscle physiology and during postmortem aging events in carcasses. *Adv. Food Res.* **1987**, *31*, 343−451.

Barrett, A. J. A new assay for cathepsin B1 and other thiol proteinases. *Anal. Biochem.* **1972**, *47*, 280−293.

Bryant, J.; Green, T. R.; Gurusaddaiah, T.; Ryan, C. A. Proteinase inhibitor II from potatoes: Isolation and characterization of its protomer components. *Biochemistry* **1976**, *15*, 3418−3423.

Chang-Lee, M. V.; Pacheco-Aguilar, R.; Crawford, D. L.; Lampila, L. E. Proteolytic activity of surimi from Pacific whiting (*Merluccius productus*) and heat-set gel texture. *J. Food Sci.* **1989**, *54*, 1116−1119, 1124.

Folk, J. E. Transglutaminases. *Annu. Rev. Biochem.* **1980**, *49*, 517−531.

García-Carreño, F.; Dimes, L. E.; Haard, N. F. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.* **1993**, *214*, 65−69.

Hamann, D. D.; Amato, P. M.; Wu, M. C.; Foegeding, E. A. Inhibition of modori (gel weakening) in surimi by plasma hydrolysate and egg white. *J. Food Sci.* **1990**, *55*, 665−669, 795.

Hass, G. M.; Venkatakrishnan, R.; Ryan, C. A. Homologous inhibitors from potato tubers of serine endopeptidases and metallocarboxypeptidases. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1941−1944.

Izquierdo-Pulido, M. L.; Haard, T. A.; Hung, J.; Haard, N. F. Oryzacystatin and other proteinase inhibitors in rice grain: Potential use as a fish processing aid. *J. Agric. Food Chem.* **1994**, *42*, 616−622.

Jiang, S. T.; Lee, J. J. Purification, characterization, and utilization of pig plasma factor XIIIa. *J. Agric. Food Chem.* **1992**, *40*, 1101−1107.

Kaiser, K. P.; Belitz, H. D. Specificity of potato isoinhibitors towards various proteolytic enzymes. *Z. Lebensm. Unters. Forsch.* **1973**, *151* (1), 18−22.

Kassell, B. Proteinase inhibitors from egg white. *Methods Enzymol.* **1970**, *19*, 890−906.

Kim, S. H.; Carpenter, J. A.; Lanier, T. C.; Wicker, L. Polymerization of beef actomyosin induced by transglutaminase. *J. Food Sci.* **1993**, *58*, 473−474, 491.

Kirschke, H.; Langner, J.; Wiederanders, B.; Ansorge, S.; Bohley, P. Cathepsin L: A new proteinase from rat-liver lysosomes. *Eur. J. Biochem.* **1977**, *74*, 293−301.

Kirschke, H.; Kembhavi, A. A.; Bohley, P.; Barrett, A. J. Action of rat liver cathepsin L on collagen and other substrates. *Biochem. J.* **1982**, *201*, 367−372.

Kishi, H.; Nozawa, H.; Seki, N. Reactivity of muscle transglutaminase on carp myofibrils and myosin B. *Nippon Suisan Gakkaishi* **1991**, *57*, 1203−1210.

Koga, H.; Yamada, H.; Nishimura, Y.; Kato, K.; Imoto, T. Comparative study on specificities of rat cathepsin L and papain: Amino acid differences at substrate-binding sites are involved in their specificities. *J. Biochem.* **1990**, *108*, 976−982.

Lin, T. S.; Lanier, T. C. Properties of an alkaline protease from the skeletal muscle of Atlantic croaker. *J. Food Biochem.* **1980**, *4*, 17−28.

Lorier, M. A.; Aitken, B. L. Method for treating fish with alpha-2-macroglobulin. U.S. Patent 5,013,568, 1991.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 256−275.

Makinodan, Y.; Yokoyama, Y.; Kinoshita, M.; Toyohara, H. Characterization of an alkaline proteinase of fish muscle. *Comp. Biochem. Physiol.* **1987**, *87B*, 1041−1046.

Mares, M.; Meloun, B.; Pavlik, M.; Kostka, V.; Bandys, M. Primary structure of cathepsin D inhibitor from potatoes and its structure relationship to soybean trypsin inhibitor family. *FEBS Lett.* **1989**, *251*, 94−98.

Masaki, T.; Shimomukai, M.; Miyauchi, Y.; Ono, S.; Tuchiya, T.; Mastuda, H.; Akazawa, H.; Soejima, M. Isolation and characterization of the protease responsible for jellification of Pacific hake muscle. *Nippon Suisan Gakkaishi* **1993**, *59*, 683−690.

Melville, J. C.; Ryan, C. A. Chymotrypsin inhibitor I from potatoes. *J. Biol. Chem.* **1972**, *247*, 3445−3453.

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Morrissey, M. T.; Wu, J. W.; Lin, D. D.; An, H. Effect of food grade protease inhibitor on autolysis and gel strength of surimi. *J. Food Sci.* **1993**, *58*, 1050−1054.

Nagase, H.; Brew, K.; Harris, E. D., Jr. Ovostatin: A proteinase inhibitor in egg white that is homologous to α_2-macroglobulin. In *Intracellular Protein Catabolism*; Alan R. Liss: New York; 1985; pp 283−285.

Osuga, D. T.; Feeney, R. E. Egg proteins. In *Food* Proteins; Whitaker, J. R., Tannenbaum, S. R., Eds.; Avi Publishing Company, Inc.: Westport, CT; 1977; Chapter 8, pp 209−267.

Porter, R. W.; Koury, B. J.; Kudo, G. Method for treating fish meat contaminated with sporozoa and potato product for improving heat gelation of fish muscle. U.S. Patent 4,935,192, 1990.

Porter, R.; Koury, B.; Kudo, G. Inhibition of protease activity in muscle extracts and surimi from Pacific whiting, *Merluccius productus*, and arrowtooth flounder, *Atheresthes stomias*. *Marine Fish. Rev.* **1993**, *55* (3), 10−15.

Reppond, K. D.; Babbitt, J. K. Protease inhibitors affect physical properties of arrowtooth flounder and walleye pollock surimi. *J. Food Sci.* **1993**, *58*, 96−98.

Rowan, A. D.; Brzin, J.; Buttle, D. J.; Barrett, A. J. Inhibition of cysteine proteinases by a protein inhibitor from potato. *FEBS Lett.* **1990**, *269*, 328−330.

Ryan, C. L. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **1974**, *249*, 5495−5499.

Seymour, T. A.; Morrissey, M. T.; Peters, M. Y.; An, H. Purification and characterization of Pacific whiting proteases. *J. Agric. Food Chem.* **1994**, *42*, 2421−2427.

Smith, C.; Megen, W. V.; Twaalfhoven, L.; Hitchcock, C. The determination of trypsin inhibitor levels in foodstuffs. *J. Sci. Food Agric.* **1980**, *31*, 341−350.

Sottrup-Jensen, L.; Borth, W.; Hall, M.; Quigley, J. P.; Armstrong, P. B. Sequence similarity between α_2-macroglobulin from horseshoe crab, *Limulus polyphemus*, and proteins of the α_2-macroglobulin family from mammals. *Comp. Biochem. Physiol.* **1990**, *96B*, 621−625.

Starkey, P. M.; Barrett, A. J. α_2-Macroglobulin, a physiological regulator of proteinase activity. In *Proteinases in Mammalian Cells and Tissues*; Elsevier/North-Holland: New York; 1977; Chapter 16, pp 663−696.

Stevens, L. Egg white proteins. Mini-review. *Comp. Biochem. Physiol.* **1991**, *100B*, 1−9.

Talley, K. Stats pack. *Pacific Fishing* **1995**, March, 55−79.

Wasson, D. H.; Reppond, K. D.; Babbitt, J. K.; French, J. S. Effects of additives on proteolytic and functional properties of arrowtooth flounder surimi. *J. Aquat. Food Prod. Technol.* **1992a**, *1* (3/4), 147−165.

Wasson, D.; Babbitt, J. K.; French, J. S. Characterization of a heat stable protease from arrowtooth flounder; *Atheresthes stomias*. *J. Aquat. Food Prod. Technol.* **1992b**, *1* (3/4), 167−182.

Weber, K.; Osborn, M. The reliability of molecular weight determinations by dodecyl/sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **1969**, *244*, 4406−4412.

Weerasinghe, V. C.; An, H. Unpublished data. Oregon State University Seafood Laboratory, Astoria, OR, 1995.

White, A.; Handler, P.; Smith, E. L. Blood. In *Principles of Biochemistry* 5th ed.; McGraw-Hill: New York; 1973; Chapter 30, pp 801−833.

Yamashita, M.; Konagaya, S. Participation of cathepsin L into extensive softening of the muscle of chum salmon caught during spawning migration. *Nippon Suisan Gakkaishi* **1990**, *56*, 1271−1277.

Yamashita, M.; Konagaya, S. Hydrolytic action of salmon cathepsin B and L to muscle structural proteins in respect of muscle softening. *Nippon Suisan Gakkaishi* **1991**, *57*, 1917−1922.