THE EFFECT OF PROTEASE INHIBITORS ON PROTEOLYSIS IN PARASITIZED PACIFIC WHITING, *MERLUCCIUS PRODUCTUS*, MUSCLE

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ABSTRACT

Since the enactment of the Fishery Conservation and Management Act of 1976, the U.S. fishing industry has intensified its interest in Pacific whiting, *Merluccius productus*, as an additional food resource. In some fishing areas, Pacific whiting is infected with a protozoan parasite, *Myxosporidia kudoa*, which produces a proteolytic enzyme that degrades the textural quality of muscle as it is processed or cooked.

Several enzyme inhibitors were evaluated for their potential to inactivate the enzyme, thereby preserving the texture of the fish during processing. It was found that protease inhibitors such as those found in egg white, potato, and soy and lima beans were ineffective as inhibitors. Compounds that react with sulfhydryl groups, on the other hand, were found to be active inhibitors. These compounds include hydrogen peroxide (free and alkaline), potassium bromate, iodoacetate, and N-ethylmaleimide. The most promising results were obtained with potassium bromate or combinations of dibasic phosphate peroxide and potassium bromate. These reagents mixed into ground parasitized pacific whiting muscle inhibited proteolysis sufficiently during frozen storage and later cooking to maintain texture comparable with nonparasitized fish.

The Fishery Conservation and Management Act of 1976 has intensified the interest of the fishing industry in Pacific whiting, *Merluccius productus*, as an additional food resource. Although Pacific whiting has been extensively fished by the Russian and Polish fishing fleets, it has attracted only slight commercial interest in the United States, primarily because its texture and color are somewhat less desirable than that of other gadoid species such as cod and haddock. In 1970, Dassow et al. observed that the textural change in cooked Pacific whiting was due to the presence of a protozoan parasite, *Myxosporidia kudoa*. This parasite produces a proteolytic enzyme capable of breaking the chemical bonds of the muscle fibers which are responsible for the characteristic texture of fresh fish. The activity of the enzyme increases as the temperature increases. Thus, during conventional processes such as baking, broiling, or pan frying, the gradual increase in heat enhances proteolysis until the product reaches the temperature of inactivation of the enzyme. One method of handling the problem of the parasitic enzyme is rapid cooking (deep-fat frying of sticks and portions) where the temperature of inactivation is achieved before proteolysis destroys the texture of the fish (Patashnik et al.[2]). Another possibility would be to inactivate the enzyme with an inhibitor.

In the work presented here, several enzymic inhibitors were evaluated to determine their effectiveness in inhibiting proteolysis in Pacific whiting muscle. The concentration of enzyme inhibitor sufficient to prevent organoleptic textural alteration was also determined.

METHODS

Pacific whiting were caught off the coast of Astoria, Oreg., by commercial trawlers, filleted and frozen within 24 h, and stored at −20°C.

The presence of the parasite was determined directly by visual evidence of black and white spores, by microscopic identification of the spores, or, indirectly, by baking a segment of muscle in a covered container for 20 min at 162°C. Soft or mushy muscle indicated the presence of the parasitic enzyme.

To ascertain the effects of enzyme inhibitors under uniform conditions, tests for proteolytic activity were carried out on diluted blends of fish

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[2]Patashnik, M., H.S. Groninger, H. Barnett, G. Kudo, and B. Koury. 1981. Pacific coast whiting (*Merluccius productus*). I. Abnormal muscle texture caused by myxosporidian-induced proteolysis. In prep., 34 p. Northwest and Alaska Fisheries Center, Natl. Mar. Fish. Serv., NOAA, 2725 Montlake Blvd. E., Seattle, WA 98112.

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muscle and on ground (minced) muscle. Conditions for testing were kept close to those under which we knew the parasitic enzyme functioned. The pH was maintained at that of the fish (6.8), the substrate was the fish muscle, and the temperature was moderate (45°C).

Blended Fish

Blended fish muscle was prepared by blending two parts 0.1 M NaCl with one part ground fish in a Lourdes Blender[3] in a quantity large enough to serve for several tests. The pH (6.8) of the solutions of the various potential inhibitors was maintained by the addition of dilute NaOH or HCl. In a 50 ml polycarbonate tube, 2 ml of the blended fish was mixed with 1 ml 0.1 M NaCl, as a control, or with 1 ml of the potential inhibitor. The tubes, covered with parafilm, were incubated for 90 min at 45°C. Duplicate samples of the control and test material were kept at 0°C in order to know the soluble protein level before incubation. This figure was subtracted from the quantity of soluble protein that was the result of increased proteolysis in the incubated sample. The reaction was stopped by the addition of 3 ml of 10% trichloroacetic acid. After 30 min at room temperature, the tubes were centrifuged at 9,750 g for 10 min. Protein determinations by the Lowry method (Lowry et al. 1951) were done on 1 ml of the supernatant. The effectiveness of the inhibitor was gauged by comparison of the proteolysis of the control (0.1 M NaCl) with that of the potential inhibitor. Since over a period of time the amount of proteolysis was bound to vary, a control was run with each experiment. In order to calculate the amount of inhibition, an arbitrary figure of 100% was assigned to the control and the effectiveness of the inhibitor was expressed as percent inhibition by the following formula:

$$\frac{\text{g protein/ml of test}}{\text{g protein/ml of control}} \times 100 = \%\ \text{proteolysis}$$

$$100 - \%\ \text{proteolysis} = \%\ \text{inhibition.}$$

Ground Fish

Ground fish was prepared by putting partially frozen fillets through a 4mm die. Ten parts of ground fish were thoroughly mixed with 1 part of 0.1 M NaCl or the inhibitor solution. Three grams of this material was incubated in a 50 ml covered polycarbonate tube for 30 min at 45°C. The reaction was stopped by the addition of 3 ml of 10% trichloroacetic acid. The remaining treatment was the same as with the blended fish.

Preparation of Ground Fish Blocks for Storage

A quantity (about 200 g) of the ground parasitized Pacific whiting was mixed with 0.1 M NaCl (approximately the ionic strength of muscle) as a control or an inhibitor in the ratio of 10 parts fish to 1 part solution. Before the blocks were placed in storage, aliquots were taken to test for inhibition and inhibitor residues. The blocks (3″ × 1″ × 8″) were stored at −20°C for 1 mo. At the end of the month, aliquots were retested for inhibition and inhibitor residues.

Effect of Proteolytic Inhibition on Texture

The blocks of parasitized whiting made for the storage study and a similar block made from nonparasitized Pacific whiting were used to test the effectiveness of maintaining texture by inhibiting proteolysis. Duplicate portions (3″ × 1″ × ½″) were cut from each block and baked in a covered dish (3½″ × 2″ × 1½″). The baked portions were randomly mixed before presenting them to an experienced panel for texture and organoleptic evaluation. In order to express the results in numerical values, numbers were assigned to the texture categories: firm (1); soft (2); mushy (3). Aliquots were taken at the same time to test for percent inhibition.

Oxidative Effect on Amino Acids

Amino acid analyses, using the Beckman 118 CL Amino Acid Analyzer (Spackman et al. 1958), were done on acid hydrolysates of nonparasitized fish, parasitized fish with no treatment, and parasitized fish which had been treated with either 0.5% disodium phosphate peroxide plus 0.025% potassium bromate or 0.5% dipotassium phosphate peroxide plus 0.025% potassium bromate.

Enzyme Inhibitors

All chemicals were of reagent grade. Trypsin

[3]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

inhibitors were purchased from Sigma Company. Dibasic phosphate peroxides were prepared in our laboratory according to the method of Nakatani and Katagiri (1970). The potato extract was prepared in our laboratory according to the method of Melville and Ryan (1972).

Test for the Presence of Peroxides or Bromates

The following method of measuring peroxides and bromates was adapted from two methods, that of Price and Lee (1970) and that of the Association of Official Analytical Chemists handbook (1975):

4 ml H_2O
1 ml of oxidant standard or 1 g fish
1 ml saturated KI
1 ml 0.001 M ammonium molybdate in 1 N H_2SO_4.

Shake for 1 min, titrate to a light yellow with 0.1 N sodium thiosulfate, and add a few drops of 1% starch; continue titrating to the end point. Both hydrogen peroxide and potassium bromate liberate iodine by oxidation; therefore, this method can be used to indicate the presence of either one. Quantification was determined by comparison with a known standard expressed in milliequivalents.

RESULTS AND DISCUSSION

Tests with Blended Fish

Blended fish was used to test a variety of potential inhibitors which are listed with concentrations and results in Table 1. The enzyme inhibitors tested included trypsin inhibitors from four sources: soybeans, lima beans, turkey egg white, and chicken egg white. We also tested crude potato extract which has been shown to contain several protease inhibitors (Melville and Ryan 1972; Ryan et al. 1974; Bryant et al. 1976; Hass et al. 1976). None of the tested enzyme inhibitors caused significant inhibition in concentrations that would be suitable for use in food systems.

From the remaining potential inhibitors which included metal chelators, oxidizers, and sulfhydryl binding compounds, we found hydrogen peroxide, potassium bromate, dibasic phosphate peroxides, iodoacetate, and N-ethylmaleimide to warrant further investigation. The reaction with iodoacetate and N-ethylmaleimide indicated that we were dealing with a thiol enzyme.

TABLE 1.—Protease inhibitors.

Inhibitor	Concentration	Active site	Effect[1]
EDTA	0.3×10^{-1} M	Chelates, Metals	±
	0.3×10^{-3} M		+
	0.3×10^{-5} M		+
Sodium pyrophosphate	0.3×10^{-1} M	Mg, Mn, Zn, other	−
	0.3×10^{-3} M	metals	±
	0.3×10^{-5} M		±
Sodium oxalate	0.3×10^{-1} M	Ca, Mg	±
	0.3×10^{-3} M		−
Cysteine	0.3×10^{-1} M	Fe, Cu, other metals	+
	0.3×10^{-3} M		±
	0.3×10^{-5} M		±
o-Phenanthroline	0.3×10^{-2} M	Fe, Co, Zn, other	−
	0.3×10^{-4} M	metals	±
Sodium fluoride	0.3×10^{-1} M	Mg, Ca, other metals	−
	0.3×10^{-3} M		±
Iodoacetate	0.3×10^{-1} M	Sulfhydryls, imid-	−
	0.3×10^{-2} M	azoles, thio ethers	−
	2.0×10^{-2} M		−
N-ethylmaleimide	0.3×10^{-2} M	Sulfhydryls	−
	1.5×10^{-2} M		−
	0.75×10^{-2} M		−
Hydrogen peroxide	1.0%	Oxidizes	−
	0.1%		−
	0.5%		−
Disodium phosphate peroxide	0.3%	Oxidizes	−
	0.5%		−
Dipotassium phosphate peroxide	0.3%	Oxidizes	−
	0.5%		−
Potassium bromate	0.05%	Oxidizes	−
	0.025%		−
	0.001%		−
Soybean	1 mg/ml	Trypsin	±
Lima bean	5 mg/ml	Trypsin	±
Chicken egg white	5 mg/ml	Trypsin	±
Turkey egg white	5 mg/ml	Trypsin	±
Potato extract	2.5 mg/ml	Chymotrypsin	±
	5.0 mg/ml	Carboxypeptidase	±
	10.0 mg/ml	Serine endopeptidase	±
		Metallocarboxypeptidase	±

[1]Increased proteolysis +, decreased proteolysis −, no signficant change ±.

Tests with Ground Fish

Both hydrogen peroxide (H_2O_2) and potassium bromate ($KBrO_3$) are currently being used in the U.S. food industry to impart desired functional and organoleptic properties to the foods to which they are added. For example, $KBrO_3$ is used in breadmaking to improve the physical properties of the dough (Tsen 1968). H_2O_2 has been used as a preservative in dairy products (Cuq et al. 1973) and as a bleaching agent in some fish products (Sims et al. 1975; James and McCrudden 1976). The dibasic phosphate peroxides have been used as a stablilizer for H_2O_2 in various food products such as soy products, meat, fish, and cereals (Pintauro 1974).

After testing for inhibition effects in the (model) blended system, tests were conducted on ground (minced) parasitized Pacific whiting to test those which demonstrated inhibitory potential and could be used in food systems.

Hydrogen Peroxide

In the ground parasitized Pacific whiting, hydrogen peroxide was significantly less effective in inactivating the proteolytic enzyme than it had been with the blended fish. This was explained by the fact that catalase is known to be present in muscle to destroy hydrogen peroxide formed in aerobic muscle fiber (Deisseroth and Dounce 1970). There was a difference between the blended and ground muscle both in protein concentration and distribution of the catalase. In order to demonstrate the difference more specifically, we compared the protein concentration and the catalase activity in the two systems. Proteins were determined by the macro-Kjeldahl, percent protein N method. Catalase activity was determined by measuring the disappearance of peroxide residues after 0.3% H_2O_2 (0.146 meq) was mixed with 1 g of blended or ground fish. The results in Table 2 show 40% less protein, which includes catalase, in blended fish than in ground fish. When 0.3% H_1O_2 was added to the blended fish, hydrogen peroxide was more slowly degraded and thereby had longer contact time with the enzyme of the parasite. The location of the catalase was shown by washing out all intercellular catalase from ground muscle, then reinstituting the catalase activity by crushing or manipulating the washed muscle fibers. A concentration of 3% H_2O_2 was needed to counteract all catalase activity, but a concentration of this magnitude also destroyed the tissue structure. It was obvious that hydrogen peroxide alone would be impractical to use as a protease inhibitor.

Potassium Bromate

Because of the difference in protein concentration in ground fish, it was necessary to increase the concentration of potassium bromate from 0.01% to 0.05% in order to achieve a 63-66% inhibition of proteolytic activity. This was shown to be sufficient to maintain the texture of parasitized Pacific whiting.

Tsen (1968) suggested that there was a synergistic effect between potassium bromate, a slow oxidizer, and faster oxidizers such as iodates, acetone peroxide, or azodecarbonamide; therefore, potassium bromate was tested with hydrogen peroxide in varying concentrations. The results were not synergistic but 0.025% $KBrO_3$ with 0.5% H_2O_2 was as effective as 0.05% $KBrO_3$ (Table 3).

TABLE 2.—Comparison of protein concentration and peroxide residues in blended or ground parasitized Pacific whiting.

Treatment of fish	Percent protein N	% peroxide residues remaining	
		0 time	5 min
Blended fish	9.88	100 (0.146 meq)	28 (0.041 meq)
Ground fish	16.51	76 (0.110 meq)	9 (0.010 meq)

TABLE 3.—Effect of hydrogen peroxide and potassium bromate on proteolysis in ground parasitized Pacific whiting.

Oxidant	% inhibiton
Control—no treatment	0
0.5% H_2O_2	43
0.05% $KBrO_3$	63
0.025% $KBrO_3$	47
0.01% $KBrO_3$	35
0.05% $KBrO_3$ in 0.5% H_2O_2	66
0.025% $KBrO_3$ in 0.5% H_2O_2	64

Dibasic Phosphate Peroxides

The adduct of hydrogen peroxide with dibasic phosphates has been found to facilitate the use of hydrogen peroxide by stabilizing it in food systems (Pintauro 1974). It seemed possible that these compounds might protect hydrogen peroxide from catalase long enough for it to be effective in inhibiting proteolysis. We tested 0.3% and 0.5% of both disodium phosphate peroxide ($Na_2HPO_4 \cdot H_2O_2$) and dipotassium phosphate peroxide ($K_2HPO_4 \cdot H_2O_2$) with ground parasitized Pacific whiting. When these compounds were compared in terms of milliequivalents of peroxides with equivalent concentrations of hydrogen peroxide alone, disodium phosphate peroxide had 23% milliequivalents of peroxide and dipotassium phosphate peroxides 15%. The dipotassium phosphate peroxide seemed less stable than disodium phosphate peroxide judging from its effervescence. Both dibasic phosphate peroxides were tested alone and with potassium bromate (Table 4). As found earlier in combination with hydrogen peroxide, 0.025% $KBrO_3$ enhanced the proteolytic inhibition of both concentrations of dibasic phosphate peroxides which meant effective inhibition could be achieved with lower concentrations of each of the oxidants.

The results of testing these inhibitors established concentrations and combinations which

TABLE 4.—Effect of dibasic phosphate peroxide on proteolysis in ground parasitized Pacific whiting.

Oxidant	% Inhibition
Control—no treatment	0
0.3% $Na_2HPO_4 \cdot H_2O_2$	35
0.3% $K_2HPO_4 \cdot H_2O_2$	9
0.3% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	64
0.3% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	62
0.5% $Na_2HPO_4 \cdot H_2O_2$	45
0.5% $K_2HPO_4 \cdot H_2O_2$	24
0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	73
0.5% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	67

were effective in inactivating the parasitic enzyme in parasitized Pacific whiting. We then determined whether 1) the inactivation would be maintained during a freeze-thaw cycle after 1 mo of storage at −20°C, 2) inactivation was sufficient to maintain a desirable texture, and 3) the treatment with oxidizing agents would adversely affect the amino acids, thereby decreasing the nutritional quality of the protein.

Effect of Frozen Storage

The prolonged effect of frozen storage on inhibition was determined on samples of ground parasitized Pacific whiting treated with various inhibitors. Aliquots of these samples were tested at the time of preparation for percent inhibition and the presence of oxidant residues. All samples were stored at −20°C for 1 mo at which time these tests were repeated, and as the results show in Table 5 there was no decrease in the inhibition of proteolysis. The ground fish treated with 0.5% H_2O_2 had no detectable residues even immediately after treatment, but maintained the inactivity of the enzyme. The residual bromate was dependent on concentration. The samples containing 0.025% and 0.05% $KBrO_3$ still had slight amounts of bromate. Bushuk and Hlynka (1960) reported that 80 ppm of bromate in bread dough disappeared completely after baking for 20 min. We baked portions of ground fish, treated with 0.05% $KBrO_3$, for 20 min at 162°C. There were no detectable residues indicating there would not be significant residues in normally cooked fish.

Effect of Inhibition on Texture

Results of the organoleptic evaluation for texture are shown in Table 6. These results demonstrate that there is a correlation between the percentage of inhibition and the maintenance of firm texture. Samples which had the highest inhibition were judged to have texture comparable with nonparasitized fish.

Oxidative Effect on Amino Acids

Some amino acids are susceptible to oxidation, particularly methionine which is readily oxidized to methionine sulfoxide and, under severe conditions, to methionine sulfone. We were using relatively mild conditions compared with other investigators, but we lacked information on the effect of potassium bromate or the combination of potassium bromate and hydrogen peroxide. We therefore compared the amino acid profiles of acid hydrolysates of nonparasitized Pacific whiting, parasitized with no treatment, and two samples of parasitized ground fish, one of which was treated with 0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$, the other with 0.5% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$. We compared the profiles for differences that might suggest significant destruction of any of the amino acids. Acid hydrolysis converts methionine sulfoxide to methionine so a difference would only show if methionine were converted to methionine sulfone. No significant differences were found in any of the amino acids (Table 7).

TABLE 5.—Storage study of oxidants in ground parasitized Pacific whiting.

Oxidant	% inhibition		Oxidant residue	
	0 time	1 mo	0 time	1 mo
Control—no treatment	0	0	0	0
0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	73	81	+[2]	+
0.5% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	67	75	+	+
0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.01% $KBrO_3$	62	59	+	N.D.[3]
0.5% $K_2HPO_4 \cdot H_2O$ + 0.01% $KBrO_3$	37	46	+	N.D.
0.5% H_2O_2	49	52	N.D.	N.D.
0.05% $KBrO_3$	66	66	+	+
0.5% H_2O_2 + 0.025% $KBrO_3$	63	69	+	+
0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.5% H_2O_2	34	47	+	N.D.

[1]Storage at −20°C.
[2]+ = presence of residue oxidant.
[3]N.D. = not detectable.

TABLE 6.—Texture evaluation of treated parasitized Pacific whiting.

Sample and treatment	Texture evaluation	% inhibition
Nonparasitized Pacific whiting	[1]1.1	
Parasitized Pacific whiting—no treatment	2.6	
Parasitized Pacific whiting treated with 0.5% H_2O_2	2.6	13
Parasitized Pacific whiting treated with 0.05% $KBrO_3$	1.1	69
Parasitized Pacific whiting treated with 0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	1.4	63
Parasitized Pacific whiting treated with 0.5% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$	1.8	64
Parasitized Pacific whiting treated with 0.5% $K_2HPO_4 \cdot H_2O_2$	2.8	28

[1]Categories: 1 = firm, 2 = soft, 3 = mushy.

Treatment of Fillets

Since a large portion of any food fish such as Pacific whiting is sold in the form of fillets, it would be preferable to treat the fillets as well as the minced fish. Recently Spinelli[4] reported on the use of adding aqueous additives into fillets by high pressure injection. The work showed that it is possible to disperse precisely given amounts of aqueous additives into fillets taken from several species of fish.

SUMMARY

The proteolytic activity in minced parasitized Pacific whiting can be effectively inhibited by the addition of hydrogen peroxide, potassium bromate, dibasic phosphate peroxides, iodoacetate, and N-ethylmaleimide. In human food systems, the only acceptable compounds of those mentioned to achieve this inhibition are hydrogen peroxide, potassium bromate, or the dibasic phosphate peroxides. The most effective inhibitors at low concentrations were 0.05% $KBrO_3$ and either 0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$ or 0.5% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$. These inhibitors retained their inhibitory effect during 1 mo of storage at −20°C. The inhibition was sufficient to maintain a firm texture when portions of the treated ground parasitized Pacific whiting were cooked. Catalase in whiting muscle rapidly degraded added hydrogen peroxide, but did not destroy potassium bromate; however, potassium bromate was reduced to undetectable levels when the material was cooked.

TABLE 7.—Percent of amino acid in hydrolysate of ground Pacific whiting muscle.

Amino acid	Non-parasitized	Nontreated parasitized	0.5% $Na_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$ treated	0.5% $K_2HPO_4 \cdot H_2O_2$ + 0.025% $KBrO_3$ treated
Aspartic acid	9.4	9.5	9.6	9.6
Threonine	4.5	4.9	4.6	4.6
Serine	4.8	5.0	5.0	5.0
Glutamic acid	13.6	13.6	13.8	13.8
Proline	3.3	3.4	3.4	3.6
Glycine	7.2	7.0	7.0	7.0
Alanine	8.8	8.4	8.7	8.5
Valine	5.6	5.5	5.6	5.5
Methionine	2.6	2.7	2.6	2.7
Isoleucine	4.2	4.2	4.2	4.2
Leucine	7.6	7.6	7.7	7.6
Tyrosine	2.2	2.2	1.9	2.2
Phenylalanine	2.8	2.8	2.8	2.8
Histidine	1.6	1.6	1.6	1.6
Lysine	7.7	7.8	7.8	7.8
Arginine	4.0	3.9	4.0	4.0

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