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Green Tea Polyphenols Inhibit Metalloproteinase Activities in the Skin, Muscle, and Blood of Rainbow Trout

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We have investigated the inhibitory effects of polyphenols from natural products, such as green tea, bilberry, grape, ginkgo, and apple, on rainbow trout gelatinase activities. Gelatinases from the skin, muscle, and blood of rainbow trout contained serine proteinase, metalloproteinase, and other proteinase activities as measured by gelatin zymography. The polyphenols of green tea caused the strong inhibition of some gelatinase activities when compared with those of the other products. This inhibition was quite similar to that of metalloproteinase by ethylenediaminetetraacetic acid, suggesting that the effects of green tea polyphenols on proteinase activities are specific for metalloproteinases. The major catechins of green tea polyphenols were then separated and identified by reverse-phase chromatography to be (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epicatechin. The effects of these catechins on gelatinase activities were examined; the most potent inhibitor of metalloproteinase activities was found to be EGCG. These results have indicated that green tea polyphenols including EGCG are useful for regulating metalloproteinase activities of fish meat.

KEYWORDS: Metalloproteinase; rainbow trout; gelatinase; polyphenol; green tea; (−)-epigallocatechin gallate

INTRODUCTION

Polyphenols are widely distributed in plant tissues. Over the past few decades, a considerable number of studies have been conducted on the effects of polyphenols on various physiological activities, such as antioxidative activity, carcinogenesis inhibitory effect, antihypertensive action, antibacterial action, free radical scavenging activity, and so on (*1*−*5*). Particularly, polyphenols in green tea leaves have been reported to have antitumor effects that inhibit metastasis and invasion of mouse Lewis lung carcinoma LL2-Lu3 cells (*6*) and human fibrosarcoma HT1080 cells (*7*). These effects are thought to be related to the inhibition of matrix metalloproteinases (MMPs), which are a family of zinc-dependent proteinases and are capable of degrading extracellular matrix components such as collagen, because the MMP family seems to play an essential role in the facilitation of tumor metastasis and invasion (*8*). Furthermore, it has been found that ester-type catechins of green tea polyphenols strongly suppress gelatin degradation by MMPs (*9*).

On the other hand, fish meat is well-known to contain several kinds of proteinases such as serine proteinase, metalloproteinase, and cysteine proteinase, and the quality of seafood products, such as dry-cured fish, smoke-dried fish, and surimi-based products, was affected by these proteinases (*10*−*20*). Particularly, fish meat is quickly softened during chilled storage, and the relevance of collagen to the softening of fish meat has recently been indicated by the observation that the solubility of collagen is increased during chilled storage of fish meat, suggesting the specific degradation of collagen by proteolytic action (*21*, *22*). The concern with the importance of regulating these proteinases has been growing in the fish food industry, while no regulating system has been developed because few inhibitors are useful as food additives.

In the present study, we have investigated the effects of various polyphenols on gelatinase activities using crude proteinase preparations from rainbow trout tissues. To identify gelatinase inhibitors, we have also separated catechins from green tea polyphenols and determined the inhibitory effects of each catechin component. Our results suggest that green tea polyphenols, which include (−)-epigallocatechin gallate (EGCG), inhibit the gelatinase activities of rainbow trout metalloproteinases.

MATERIALS AND METHODS

Materials. Rainbow trout, *Oncorhynchus mykiss*, were commercially bought in the Tokyo central wholesale market, Japan. Reagents for biochemical experiments were of the highest quality available from

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commercial vendors. Several polyphenol preparations from ginkgo, grape, bilberry, and Japanese green tea were from Tokiwa Phytochemical Co. (Tokyo, Japan), and those from apple were from NIKKA Co. (Tokyo, Japan). Purified caffeine and catechins, (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), and (+)-catechin, were from Funakoshi Co. (Tokyo, Japan).

Preparations of Crude Enzyme. Rainbow trout skin, muscle, and blood were separated and homogenized with two volumes of 0.25% (v/v) Triton X-100 at 4 °C. The suspension was centrifuged at 10000*g* for 20 min. The supernatant was maintained as aliquots at −80 °C until use for each analysis. The approximate protein concentration was determined by a Micro BCA Protein assay reagent kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Gelatin Zymography. Gelatinase activity was analyzed by substrate gel electrophoresis in a 10% polyacrylamide gel containing 1 mg/mL of porcine gelatin and 0.5% SDS (*23*). Aliquots of 10 or 2 μL of samples were diluted with an equal volume of Laemmli sample buffer without 2-mercaptoethanol and electrophoresed at 4 °C. After removal of SDS by washing in 0.25% (v/v) Triton X-100 for 30 min, the gel was incubated at 25 °C for 20 h with buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% (v/v) Triton X-100, and 0.02% (w/v) NaN_3. The buffer was decanted, and the gel was stained with Coomassie brilliant blue R-250 and then destained. The molecular mass of gelatinolytic components was estimated by using Prestained Protein Molecular Weight Standards (Life Technolgies, Frederick, MD). To examine an inhibitory effect, 10 mM 1,10-phenanthroline, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM diisopropyl fluoride (DFP), 20 mM leupeptin, or 27 mM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane) (E-64) was added to the incubation buffer. The other inhibitory effects were investigated by using 2 mg/mL polyphenols and 1 mM catechins. Human MMP-2 (Sigma, St. Louis, MO) was used as a control.

Quantification of Catechins from Green Tea Polyphenols. Green tea polyphenols were dissolved at a concentration of 3 mg/mL in deionized water, and tea extracts were prepared by infusing commercial green tea leaves (*Camellia sinensis*) in 10 volumes (v/w) of boiling deionized water for 2 min. Catechins were separated by reverse-phase HPLC using a TSKgel ODS-80Ts QA column of 4.6 × 250 mm (Tosoh, Tokyo, Japan) at 40 °C and were monitored by using a UV detector at 280 nm. The eluting mixture of H_2O containing 0.06% H_2SO_4, acetonitrile, and ethyl acetate (86:12:2) was used at a flow rate of 1 mL/min (*24*). Identification and quantification of each catechin were confirmed by comparison of retention time with authentic standards of EGCG, EGC, ECG, EC, and (+)-catechin.

Gelatinase Fluorometric Assay. Fluorometric assays of gelatinase activities of the crude enzyme from rainbow trout muscle were performed by using an EnzChek Gelatinase assay kit (Molecular Probes, Eugene, OR) and a fluorescence spectrophotometer F2000 (Hitachi, Tokyo, Japan). The crude enzyme (3 mg) was mixed with 50 μg of fluorescein-conjugated gelatin in a final volume of 1 mL of the reaction buffer. After incubation at 37 °C for 8 h, the reaction mixture was centrifuged at 10000*g* for 5 min, and the supernatant was used as a sample. To examine inhibitory effects, EDTA or EGCG was added in the assay system, and the rate of proteolysis, in the presence or absence of the inhibitors, was determined by measuring the increase in fluorescence.

RESULTS

Inhibitory Effects of Gelatinolytic Activities by Proteinase Inhibitors. The extracts from the skin, muscle, and blood of rainbow trout were analyzed by gelatin zymography. As shown in **Figure 1A**, the zymogram indicates multiple and variable protein bands with several gelatinolytic activities. The major activities in the extracts of skin and blood were clearly detected at the positions of about 85, 73, 67, 20, and 19 kDa and at the positions of about 110, 85, 73, and 67 kDa, respectively. The blood extracts appeared to contain a higher amount of gelatinolytic activities than the other tissue extracts. The major activities in the muscle extracts were slightly detected at the positions of about 85 and 67 kDa. The gelatinolytic activities from rainbow trout muscle were approximately similar to those of the crude enzymes from the muscle of ayu *Plecoglossus*

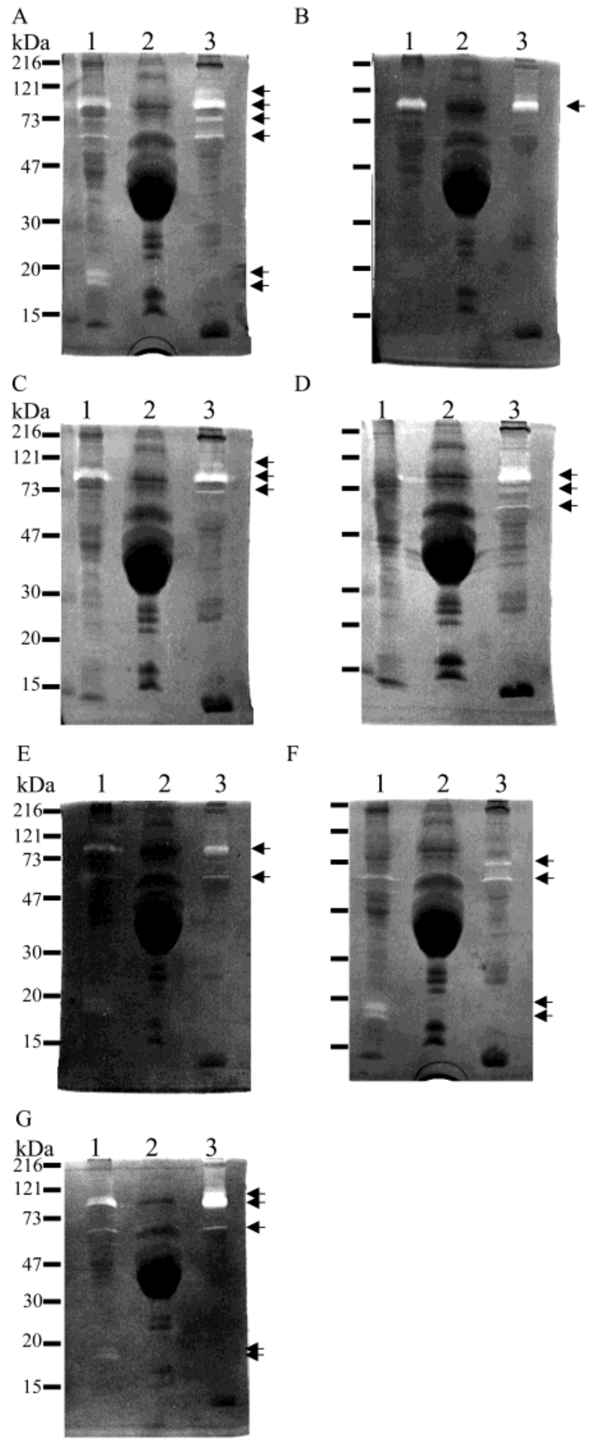

Figure 1. Effect of inhibitors on the gelatinolytic activities of tissue extracts from rainbow trout. The extracts (10 μL) of skin and muscle and the extract (5 μL) of blood were subjected to zymographic analysis (lane 1, skin; lane 2, muscle; lane 3, blood). To examine an inhibitory effect, some proteinase inhibitors were added in the incubation buffer. The incubation was performed without any inhibitors (A) and with 10 mM 1,10-phenanthroline (B), 1 mM ethylenediaminetetraacetic acid (C), 1 mM phenylmethylsulfonyl fluoride (D), 2 mM diisopropyl fluoride (E), 20 mM leupeptin (F), or 27 mM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane) (G). Arrows indicate the major gelatinolytic activities.

Table 1. Effect of Inhibitors on the Gelatinolytic Activity of Tissue Extracts from Rainbow Trout

	gelatinolytic component (kDa)	absence of inhibitor	presence of inhibitor					
			PT[a]	EDTA	PMSF	DFP	leupeptin	E-64
skin	85	+++[b]	+++	+++	−	++	−	++
	73	±	±	±	−	−	−	−
	67	+	−	−	−	±	+	+
	20	+	−	−	−	±	+	±
	19	+	−	−	−	±	+	±
muscle	85	+	±	±	−	−	−	−
	67	+	−	−	±	±	+	±
blood	110	++	±	±	±	−	−	+
	85	+++	++	++	+++	++	+	+++
	73	++	−	+	+	±	±	−
	67	++	−	−	+	+	+	+

[a] PT, 1,10-phenanthroline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluoride; E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane). [b] +, positive; −, negative.

altivelis (*25*). To classify proteinases in these tissues, several inhibitors were added to the incubation buffer (**Figure 1B**−**G**). The results suggested that some gelatinolytic activities were clearly due to various types of proteinases. **Table 1** shows the list of major activities in their extracts analyzed with inhibitors and the relative amount of activity. Both 1,10-phenanthroline and EDTA, which are known to inhibit activation of metalloproteinases such as MMPs, clearly decreased active components at 67, 20, and 19 kDa in the skin extracts, at 67 kDa in the muscle ones, and at 73 and 67 kDa in the blood ones. PMSF and DFP, which are known as inhibitors of serine proteinases, reduced gelatin lysis at 73 kDa in the skin extracts, at 85 kDa in the muscle ones, and at 110 and 73 kDa in the blood ones. As for PMSF, a 85-kDa component in the skin extracts was completely inhibited. On the other hand, inhibition of active components by leupeptin was approximately similar to that by E-64, and these two inhibitors, which are known as inhibitors of cysteine proteinases, decreased active components at 73 kDa in the skin extracts, 85 kDa in the muscle ones, and 73 kDa in the blood ones. Moreover, leupeptin completely inhibited active components at 85 kDa in the skin extracts and 111 kDa in the blood ones, and a lot of 85-kDa components in the blood extracts were also affected by leupeptin. The inhibitory difference for gelatinolytic activities between these two proteinase inhibitors suggests that a gelatinolytic active band consists of some active components and each proteinase has differential sensitivity to inhibitors.

Inhibitory Effects of Polyphenols on Gelatinolytic Activities. The inhibitory effects on gelatinolytic activities from the rainbow trout tissues were investigated by adding polyphenols from ginkgo, grape, apple, bilberry, or green tea in the gelatin zymogram (**Figure 2**). Among all the polyphenols tested, green tea polyphenols caused the strongest inhibition of gelatinolytic activities of the extracts of skin, muscle, and blood. The inhibitory patterns of green tea polyphenols are clearly similar to those of 1,10-phenanthroline and EDTA, as shown in **Figure 1**. Moreover, the inhibition of these gelatinolytic activities has a close resemblance to that of the activities of human MMP-2, suggesting the inhibition of the activities of metalloproteinases. The polyphenols from grape and bilberry showed a weak, inhibitory effect on metalloproteinases, and those from ginkgo and apple also slightly decreased the gelatinolytic activities.

Quantification of Catechins in Green Tea Polyphenols. To characterize the inhibition of metalloproteinases by green tea polyphenols, the polyphenols were subjected to HPLC analysis. EGCG, EGC, ECG, and EC are well-known as the major components of polyphenols obtained from green tea leaves (*24*). As seen in **Figure 3**, the composition of green tea

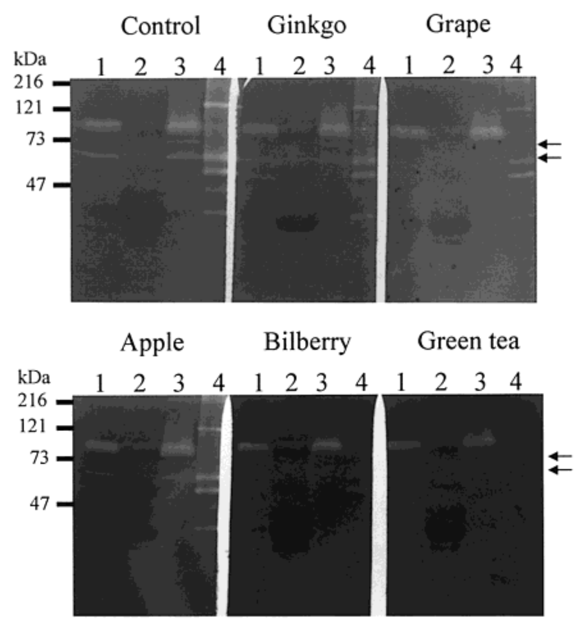

Figure 2. Effect of polyphenols on the gelatinolytic activities of tissue extracts from rainbow trout. The extracts (3 μL) of skin and muscle and the extract (1.5 μL) of blood were subjected to zymographic analysis (lane 1, skin; lane 2, muscle; lane 3, blood). The incubation was performed without any polyphenols (control) and with polyphenols from ginkgo, grape, apple, bilberry, and green. Shown is positive control incubation with 0.1 mg of human MMP-2 (lane 4). Note that two bands indicated by arrows disappear after the incubation.

polyphenols is relatively consistent with that of the extracts from Japanese green tea leaves. EGCG was the most abundant (69% of total catechins) in green tea polyphenols, followed by EGC (13%), ECG (12%), and EC (5%). The results also showed that the yields of total catechins and caffeine in green tea polyphenols were 75 and 7 g/100 g of dry powder, respectively.

Inhibitory Effects of Catechins. The inhibitory effects of the major catechins such as (+)-catechin, EGC, EC, ECG, and EGCG are examined by gelatin zymography (**Figure 4**). EGCG partially suppressed the gelatinolytic activities from the skin, muscle, and blood extracts. The inhibitory pattern of EGCG was significantly similar to those of green tea polyphenols, EDTA, and 1,10-phenanthroline and indicated the inhibition of gelatinolytic proteolysis by a 67-kDa component and human MMP-2. It was found from these results that EGCG obviously reduced the gelatinolytic activities of rainbow trout tissues as a metalloproteinase inhibitor. Both EGC and ECG showed a weak inhibitory effect, whereas (+)-catechin and EC had little effect.

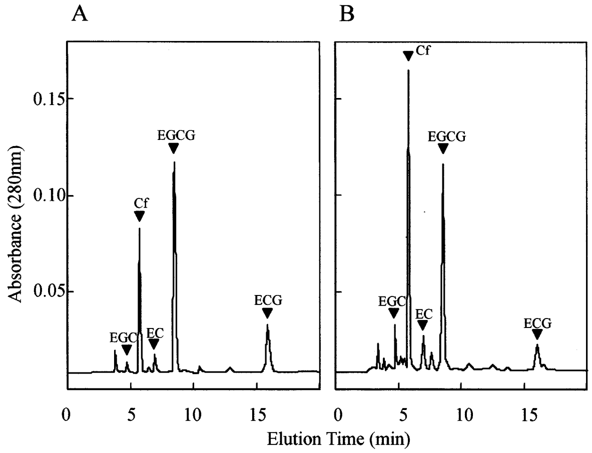

Figure 3. Reverse-phase HPLC of catechins from green tea polyphenols (A) and the extracts prepared from Japanese green tea leaves (B). Samples were loaded on a TSKgel ODS-80Ts QA column of 4.6 × 250 mm and eluted at a flow rate of 1 mL/min with the mixture of H_2O containing 0.06% H_2SO_4, acetonitrile, and ethyl acetate (86:12:2). Identification and quantification of each catechin were confirmed by comparison of retention time with authentic standards of caffeine (Cf) and catechins, (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epicatechin (EC), and (+)-catechin.

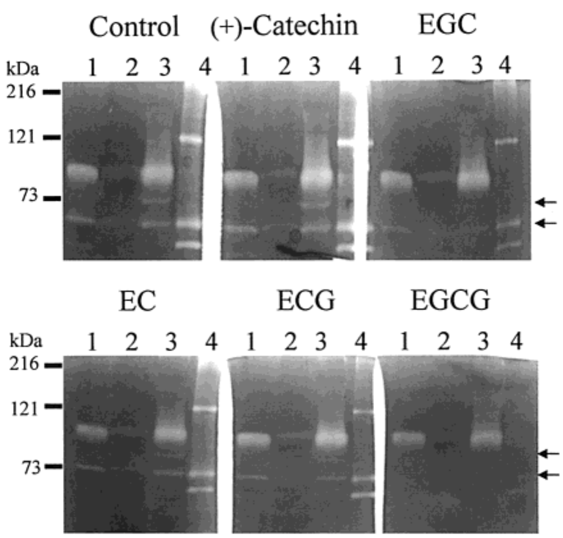

Figure 4. Effect of catechins on the gelatinolytic activities of tissue extracts from rainbow trout. The extracts (3 μL) of skin and muscle and the extract (1.5 μL) of blood were subjected to zymographic analysis (lane 1, skin; lane 2, muscle; lane 3, blood). The incubation was performed without any catechins (control) and with (+)-catechin, (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG). Shown is positive control incubation with 0.1 mg of human MMP-2 (lane 4). Note that two bands indicated by arrows disappear after the incubation.

The inhibition by EGCG was further characterized on the gelatinolytic activities of rainbow trout muscle, and the efficiency is evaluated by fluorescence assays (**Figure 5**). The gelatinolytic activity of the muscle extracts was measured in the presence of EGCG or EDTA and was expressed as the percentage against the gelatinolytic activity in the absence of inhibitors. Both EGCG and EDTA were found to decrease the gelatinolytic activities from the muscle extracts according to their concentration and to inhibit more than 40% at 0.1 mM of EGCG.

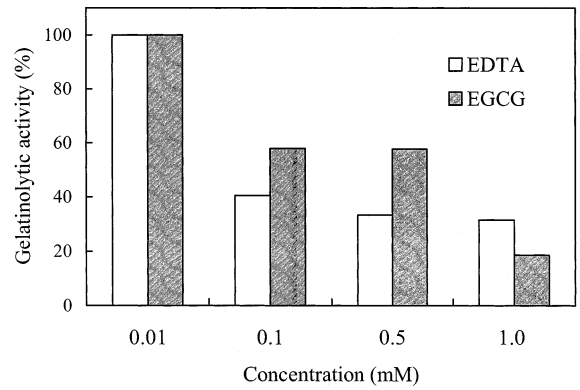

Figure 5. Effects of (–)-epigallocatechin gallate (EGCG) and ethylenediaminetetraacetic acid (EDTA) on the gelatinolytic activities of rainbow trout muscle extracts. The activities were measured by fluorometric assays in the presence of increasing concentrations of EGCG or EDTA. The results are expressed as the percentage of control activities (n = 3).

DISCUSSION

In the present study, we have investigated the effects of various polyphenols on rainbow trout proteinases by gelatin zymography. Green tea polyphenols caused the partial inhibition of gelatinase activities from rainbow trout skin, muscle, and blood tissues. The proteinase inhibition was quite similar to the inhibition of metalloproteinase activities by 1,10-phenanthroline and EDTA. Furthermore, the effects of the major catechins from green tea polyphenols were examined on gelatinase activities. The most potent inhibitor of metalloproteinase activities from the rainbow trout tissues was found to be EGCG. This is in good agreement with the findings that EGCG from green tea polyphenols strongly inhibited the activities of human MMPs-2 and -9 and mouse MMPs-9 and -12 (*6*, *7*, *9*).

Recently, fish MMPs-2, -9, and -13 have been cloned, and the distribution of these MMPs has been revealed in several tissues (*19*, *23*, *25*−*32*). In particular, rainbow trout MMP-2 mRNA has been detected in skin, muscle, and blood, and the product has been also identified as a 67-kDa component by zymography and Western blot analysis (*33*). In this study, it is quite likely that the gelatinolytic activities of these MMPs in rainbow trout tissues are almost inhibited by green tea polyphenols and EGCG.

On the other hand, the specific degradation of pericellular type V collagen, which is known as a minor extracellular matrix component, was observed during chilled storage of the muscles of rainbow trout and sardine (*21*, *22*). The specific degradation of type V collagen is thought to cause disintegration of muscle fibers and muscle softening. Moreover, MMPs-2 and -9 have been reported as being active in the cleavage of native type V collagens and denatured proteins from all types of collagen (*8*). These MMPs may be the active components that should be suppressed in fish muscle softening during chilled storage. As a feed supplement in cultured fish, green tea polyphenols have been reported to increase the amount of muscle collagen (*34*, *35*). Therefore, there is a fair possibility of having an effect on the muscle softening of fish fed by green tea polyphenols or EGCG.

The food industry has searched for effective food additives to control proteinase activity. It was reported that the proteinase activity in fish meat and surimi can be decreased by using proteinase inhibitors such as egg white, beef plasma protein, some components from plants, and a peptide from bacteria (*36*−*39*). However, most of the inhibitors reduce serine and cysteine proteinase activities and have limited commercial use because of their chemical properties, safety problems, cost, and governmental regulation. It is clear that green tea polyphenols including EGCG are useful for regulating metalloproteinase activities of fish meat. Thus, the inhibition of metalloproteinase activities and the quality control of fresh meat as a raw material of processing food are expected to be achieved by soaking the meat in the polyphenol solution and spraying the meat with the solution.

ABBREVIATIONS USED

MMP, matrix metalloproteinase; EGCG, (−)-epigallocatechin gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin gallate; EC, (−)-epicatechin; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluoride; E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane).

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