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Inhibition of mackerel (*Scomber scombrus*) muscle lipoxygenase by green tea polyphenols

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Abstract

The high polyunsaturated fatty acid content of oily fish such as mackerel (*Scomber scombrus*) makes it particularly susceptible to oxidative degradation. We have shown previously the presence of lipoxygenase (LOX), a lipid oxygenase, in mackerel muscle. In the current study, commercially available green tea polyphenols were shown to effectively inhibit the LOX activity of mackerel muscle. EGCG (epigallocatechin gallate) was the strongest inhibitor tested with an IC_{50} (concentration for half maximal inhibition) value of 0.13 nM. All the tea catechins showed a *mixed* non-competitive type inhibition. In addition, antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), esculetin, caffeic acid, ascorbic acid, and ethylene diamine tetraacetic acid (EDTA) were effective to varying degrees (IC_{50} values between 0.02 and >50 μM) in the inhibition of mackerel muscle LOX. Nordihydroguaiaretic acid (NDGA), a classical LOX inhibitor and potassium cyanide (KCN), a heme protein inhibitor were assayed for their inhibitory activities for comparison.

Post harvest spoilage of fish account for loss of as much as 10% of the world's catches of cultured fish. This data indicates that the green tea polyphenols, nature's very potent antioxidants, may be used as an effective and natural means of reducing post harvest spoilage in fish.

Keywords: Lipoxygenase; Mackerel; Inhibition; Green tea; Antioxidants

1. Introduction

Fish lipids are susceptible to oxidation owing to the high levels of polyunsaturated fatty acids (PUFA), even in frozen storage, and this can affect the flavor, texture, taste, aroma and shelf life of fish (Ke & Ackman, 1976). Since the direct interaction between oxygen and highly unsaturated lipids is kinetically hindered (Kanner, German, & Kinsella, 1987), the enzymatic initiation of oxidation by enzymes such as lipoxygenase (LOX), peroxidases and microsomal enzymes has been gaining favor.

LOX (EC 1.13.11.12), now known to be widely distributed in plants, animals and microorganisms, is a dioxygenase that oxygenates PUFA, converting them to hydroperoxides. Peroxyl radical complexes have been reported to exist during the catalytic cycle of LOX and can serve as sources of free radicals (Robinson, Wu, Domoney, & Casey, 1995). Therefore, antioxidants such as flavonoids which act as free radical quenchers (Zhou, Miao, Yang, & Liu, 2005) may act as LOX inhibitors. In addition, it has been proposed that the inhibitory effects of antioxidants depend on the physico-chemical state of the substrate and the type of LOX, and that they may change completely depending on the conditions (Noguchi et al., 2002).

With a view of the various detrimental effects of imbalances or perturbations in fatty acid oxidation, there has been considerable interest in the development of inhibitors of LOX (Casey & Hughes, 2004; Schneider & Bucar, 2005). We have previously reported the isolation and characterization of LOX from mackerel muscle (Banerjee, Khokhar, & Owusu Apenten, 2002). The aim of the current study was

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to study the inhibition of mackerel muscle LOX (mmLOX) by natural antioxidants. The most widely used inhibitors that have been reported to be effective against fish LOX are nordihydroguaiaretic acid (NDGA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), esculetin, ascorbic acid and α-tocopherol (Grün & Barbeau, 1995; Harris & Tall, 1994; Hsieh, German, & Kinsella, 1988; Mohri, Tokuori, Endo, & Fujimoto, 1999; Saeed & Howell, 2001).

Green tea polyphenols have potent free radical quenching and antioxidant activities (Wiseman, Balentine, & Frei, 1997) and have structural features that may specifically interfere with the arachidonic acid cascade, including the LOX pathway (Hong & Yang, 2003; Hussain, Gupta, Adhami, & Mukhtar, 2005). In addition, with growing concerns regarding the safety of synthetic antioxidants such as BHT and BHA, alternative mechanisms of antioxidant protection by the use of natural antioxidants have been in review over the past years (Barlow, 1990).

The current study shows that green tea polyphenols are very potent inhibitors of mmLOX, with EGCG (epigallocatechin gallate) as the most effective inhibitor (IC_{50} 0.13 nM) followed by ECG (epicatechin gallate) (IC_{50} 0.8 nM), EC (epicatechin) (IC_{50} 6.0 nM), EGC (epigallocatechin) (IC_{50} 9.0 nM) and Ct (catechin) (IC_{50} 22.4 nM). A number of other inhibitors were also assayed for comparison, including BHA, BHT, ascorbic acid, esculetin, NDGA ethylene diamine tetraacetic acid (EDTA) and potassium cyanide (KCN).

Green tea glazing was shown to improve the storage quality of frozen bonito fillets (Lin & Lin, 2005). In addition, hot water tea extract was shown to suppress the pro-oxidant activities of the dark meat and skin of blue sprat (Seto, Lin, Endo, & Fujimoto, 2005). The current study proposes that the improvement in the shelf life of fish by green tea polyphenols is at least in part due to inhibition of LOX resulting in delaying oxidation of fish lipids.

2. Materials and methods

2.1. Enzyme purification

Mackerel muscle LOX was extracted as described (Banerjee et al., 2002). Briefly, the crude enzyme extracted from a cold acetone precipitation of mackerel muscle (specific activity 0.028 μmol min^{-1} mg^{-1}) was further precipitated by ammonium sulfate fractionation at 40% and 70% saturation (specific activity 0.043 μmol min^{-1} mg^{-1}). This extract was then extensively dialyzed and partially purified by successive passage through a hydroxyapatite (HA) column followed by a Sephadex G-100 gel filtration column (Banerjee, 2002). Temperature was maintained at 0–4 °C. Briefly, the crude mmLOX (8 mL) was applied to a HA column equilibrated with sodium phosphate buffer (pH 7.0, 20 mM). Elution was carried out with a stepwise gradient of 0.2 M (pH 7.0, 70 mL) and 0.35 M (pH 7.0, 60 mL) phosphate buffer. The enzyme activity was eluted at 0.2 M buffer concentration and the specific activity was 0.093 μmol min^{-1} mg^{-1} with a 41% yield. The fractions were then concentrated by ultrafiltration with no loss in activity. The resultant enzyme fraction (5 mL, ca. 1 mg mL^{-1}) was then applied on the gel filtration column and fractionation was carried out with 300 mL phosphate buffer (0.1 M, pH 7.0) at 0.2 mL min^{-1} and fractions of 6 mL were collected. Fractions were assayed for LOX activity and the enzyme was determined to be purified at least 13-fold (specific activity 0.36 μmol min^{-1} mg^{-1}) with a 30% yield. Protein homogeneity of active fractions was examined through SDS PAGE and showed one major band and two minor bands. The molecular weight of the major band corresponded with the molecular weight of mmLOX determined by gel filtration chromatography. Protein determination was carried out by the modified Bradford method using bovine serum albumin as standard (Bradford, 1976).

2.2. Enzyme assay

Mackerel muscle LOX was assayed spectrophotometrically at 234 nm using linoleic acid as substrate as described (Banerjee et al., 2002).

2.3. Inhibition of mmLOX

Inhibition of the partially purified LOX was studied in presence of the green tea polyphenols Ct, EC, ECG, EGC and EGCG. Other inhibitors tested were NDGA, BHT, BHA, esculetin, EDTA, KCN, caffeic acid and ascorbic acid. All the compounds were commercially obtained (Sigma, UK) and stock solutions (3 M, 0.3 M or 30 mM) were prepared in water (EDTA, KCN, ascorbic acid); methanol (Ct, EC, ECG, EGC, EGCG); or ethanol (NDGA, BHT, BHA, esculetin, caffeic acid); aliquoted and stored at −20 °C. Appropriate volumes of the stock solution were added to the linoleic acid substrate (1 mM) to give the final range of concentrations studied (see Tables 1 and 3). The substrate–inhibitor solution was incubated for 2 min after which the enzyme solution was added in a final volume of 3 mL and the mixture was assayed spectrophotometrically at 234 nm. Any change in absorbance of in the substrate inhibitor mixture during the incubation period was subtracted from the final result. The percentage inhibition was expressed as

$$\{(A_0 - A_1)/A_0\} \times 100,$$

Table 1
The IC_{50} values for inhibition of mmLOX by tea catechins

Compounds	Concentrations (nM)	IC_{50} (nM)
Catechin (Ct)	6–120	22.4
Epicatechin (EC)	0.6–60	6.0
Epicatechin gallate (ECG)	0.4–7.7	0.8
Epigallocatechin (EGC)	5–110	9.0
Epigallocatechin gallate (EGCG)	0.04–0.4	0.13