Immunohistochemical Localization of Cathepsins B and L in the White Muscle of Chum Salmon (*Oncorhynchus keta*) in Spawning Migration: Probable Participation of Phagocytes Rich in Cathepsins in Extensive Muscle Softening of the Mature Salmon

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The mode of existence of cathepsins B and L in the white (ordinary) muscle of chum salmon was examined immunohistochemically with the specific antibodies vs cathepsins B and L. Cathepsins B and L clearly were demonstrated to be present in macrophage-like phagocytes near/in the muscle fibers in the white muscle. In particular, numerous phagocytes were observed around the necrotic muscle fibers. Therefore, the high cathepsin activity is considered to be brought about by the phagocytes appearing in the white muscle. It is probable that the phagocyte in the muscle takes part in the destruction of muscle fiber associated with drastic physiological change such as sexual maturation and/or starvation of the fish during spawning migration. The extensive muscle softening which is often observed in the catch of mature salmon is suggested to be due to the proteolytic degradation of the fine structure of myofibril and intracellular connective tissue by cathepsin L liberated from the phagocytes in the muscle.

INTRODUCTION

The white (ordinary) muscle of chum salmon during spawning migration shows high activities of cathepsins B, D, H, and L (Konagaya, 1982, 1985; Yamashita and Konagaya, 1990a,b). It has been assumed that this enhancement of catheptic activity is closely related to sexual maturation and starvation during the spawning migration (Konagaya, 1982, 1985; Mommsen et al., 1980). However, little is known about the cellular mechanism by which such high catheptic activity in the salmon muscle is brought about.

From the practical viewpoint of the quality of salmon, the cause of extremely soft muscle often observed in the salmon caught during spawning migration was elucidated; i.e., cathepsin L in the muscle whose activity rises remarkably along with maturation was ascertained to be the most probable enzyme responsible for the softening (Yamashita and Konagaya, 1990b). Since the salmon muscle with high catheptic activity is a useful example for investigation of the muscle autolysis occurring during storage after harvest, the histological localization of cathepsins in the muscle should prove to be interesting.

It is a generally accepted idea that cathepsins play an important role in intracellular proteolysis (Kirschke and Barrett, 1987). A marked increase of catheptic activity was observed in some pathological muscles, such as myopathy and muscle dystrophy of mammals (Goldspink and Lewis, 1987; Ishiura et al., 1983; Kominami et al., 1984), and in the involuting tissue of anuran metamorphosis (Weber, 1969). The enhancement of catheptic activity in the muscle tissue is ascribed to an increase of phagocytes rich in cathepsins. Previous studies dealing with the enhanced catheptic activity had been confined to mammalian diseases (Goldspink and Lewis, 1987; Ishiura et al., 1983; Kominami et al., 1984) and anuran metamorphosis (Weber, 1969). The high catheptic activity in the salmon muscle is of particular interest because it is responsible for the physiological changes associated with sexual maturation during the spawning migration of the fish (Yamashita and Konagaya, 1990b; Mommsen et al., 1980).

The present study was undertaken to elucidate the cause of the enhancement of catheptic activity in the white muscle of chum salmon during spawning migration. By an immunohistochemical examination using anticathepsins B and L antibodies, the cathepsins were localized in many phagocytes existing between muscle fibers. The present finding may provide also a new concept that makes clear the mechanisms of protein turnover taking place in the muscle of salmon and the extensive muscle softening, i.e., the muscle tissue degradation occurring after the death of the fish.

MATERIALS AND METHODS

Materials. Chum salmon (*Oncorhynchus keta*) during spawning migration were caught in the Kuji River, Ibaraki, Japan, in December 1989. Twelve fish taken out of a catch were instantly killed and brought back to our laboratory on ice.

Immunohistochemical Examination. Cathepsin B (Yamashita and Konagaya, 1990c) and cathepsin L (Yamashita and Konagaya, 1990b) were purified to homogeneity from the white muscle of chum salmon caught during spawning migration. Antisera against cathepsin B and cathepsin L were prepared by immunizing the purified enzymes, respectively, into two rabbits. IgG fractions were separately fractionated from the antisera by ammonium sulfate fractionation and chromatography on DEAE-Sephadex A-50 (Pharmacia) as described by Kominami et al. (1984). Each monospecific IgG to the enzymes was further purified by affinity chromatography with the enzyme coupled to Sepharose 4B (Kominami et al., 1984).

Frozen sections (10 μm thick) of the dorsal muscle of fresh chum salmon were stained immunohistochemically with the antibodies and peroxidase–antiperoxidase conjugate (Histogen kit, Seikagaku Kogyo) by the method described by the manufacturer. Positive reactions were seen with aminoethylcalvasole. All sections were counterstained with methylgreen.

Assay of Proteolytic Activity. The autolytic activity of the muscle homogenate was measured according to the method of Konagaya (1982) as follows. Specimens of the white muscle of chum salmon were homogenized with 3 volumes of cold distilled water in a Polytron homogenizer (Kinematica). A mixture of 0.5 mL of 0.1 M sodium citrate phosphate buffer (pH 6.5) and 0.5

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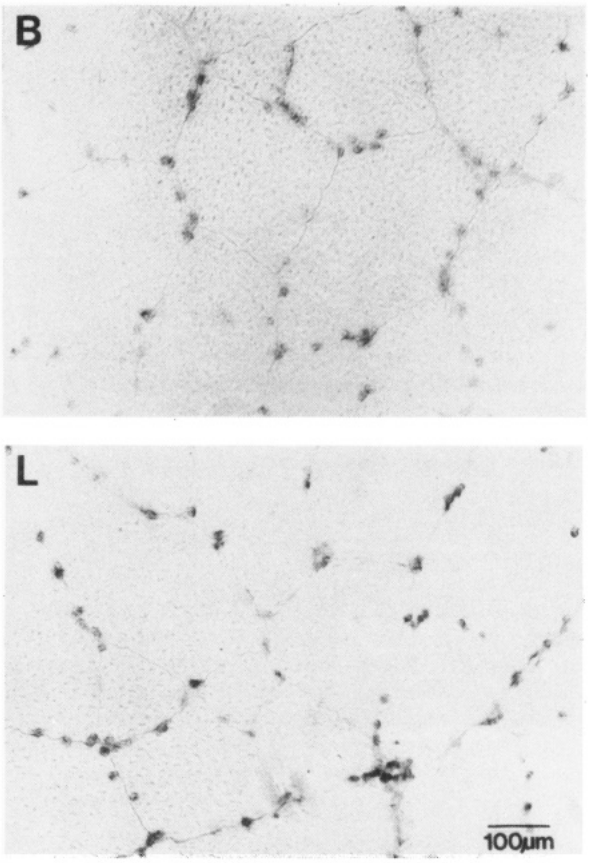

Figure 1. Immunohistochemical staining of the sections taken from the white muscle of chum salmon during spawning migration with anticathepsin B (B) and anticathepsin L (L) showing the existence of many phagocytes between muscle fibers.

mL of the homogenate was incubated at 30 °C for 1 h, and the reaction was stopped by adding 5% trichloroacetic acid at a final concentration. One unit of the activity is defined as the activity degrading 1 μg of protein/min at 30 °C.

RESULTS

The sections taken from the white muscle of chum salmon during spawning migration were stained immunohistochemically with anticathepsin B rabbit IgG and with anticathepsin L rabbit IgG. Nonmuscle cells, which were a kind of phagocyte present around muscle cells, gave strongly positive reactions to both cathepsins B and L (Figure 1).

The muscles having a large number of nonmuscle cells (220–340 cells/mm^2) and high proteolytic activity (64–89 units/g of tissue) softened to a pastelike appearance during several days of storage of the salmon (Figure 2), as was noted in a previous paper (Yamashita and Konagaya, 1990a). There is considerable variation in the number of nonmuscle cells and proteolytic activity in the muscle tissue. A good correlation was observed between the number of nonmuscle cells and proteolytic activity in the muscle of 12 fish samples (Figure 2). The higher the proteolytic activity in the muscle, the larger the number of nonmuscle cells found (Figures 2 and 3). Furthermore, in the muscle containing a large number of nonmuscle cells, necrotized muscle fibers were frequently observed, and in the vicinity of the necrotized muscle fibers, numerous nonmuscle cells were scattered (Figure 3-1).

The size of the nonmuscle cell was 10–20 μm in diameter. Many granules were observed within the nonmuscle cells by peroxidase staining and Giemsa staining (Figure 4). Positive reactions to both peroxidase and naphthylbutylate esterase activity tests revealed that the nonmuscle cells are macrophage-like cells.

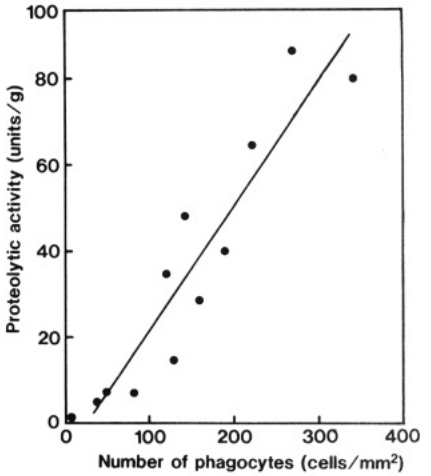

Figure 2. Relationship between the number of the phagocytes and the proteolytic activity in the salmon muscle. The correlation coefficient was calculated to be 0.93.

The present study on the localization of cathepsins B and L in the salmon muscle indicates that the high activity of cathepsins B and L in the white muscle of chum salmon in spawning migration resulted from the appearance of phagocytes rich in these enzymes in the muscle.

DISCUSSION

The present study clearly demonstrates that the high levels of cathepsins in the white muscle of chum salmon in spawning migration are attributable to the appearance of a large number of nonmuscle cells existing between muscle fibers. These nonmuscle cells gave strongly positive reactions to both cathepsins B and L and displayed the activities of peroxidase and naphthylbutylase. The nonmuscle cell in the salmon muscle is considered to be macrophage-like because macrophage-type cells were reported to contain cathepsins, other lysosomal hydrolases, and peroxidase at high levels (Ishiura et al., 1983; Kominami et al., 1984).

Macrophages rich in cathepsins are known to infiltrate the inflamed and necrotic muscle and thereby to increase the activity of acid protease. Ishiura et al. (1983) and Kominami et al. (1984) have reported that the phagocyte increases markedly the catheptic activity in the skeletal muscle of dystrophic mammals. This implies that the phagocyte in the dystrophic muscle may be responsible for the development of necrosis and degeneration of dystrophic muscle.

The infiltration of phagocytes in the skeletal muscle is observed in the involuting anuran tail muscle during metamorphosis (Weber, 1969). In this case the phagocyte is inferred to participate in the tissue destruction.

The appearance of a large number of phagocytes in the muscle of the salmon during spawning migration was ascertained by the histochemical experiment in the present study. The salmon in spawning migration is considered to utilize his own muscle protein as the energy source on starvation and sexual maturation of the fish (Mommsen et al., 1980; Yamashita and Konagaya, 1990a,b). It is, therefore, likely that the many phagocytes appearing in the muscle may participate in the destruction of the muscle fiber of salmon which is under the unusual physiological condition associated with sexual maturation and starvation during spawning migration (Mommsen et al., 1980; Yamashita and Konagaya, 1990b).

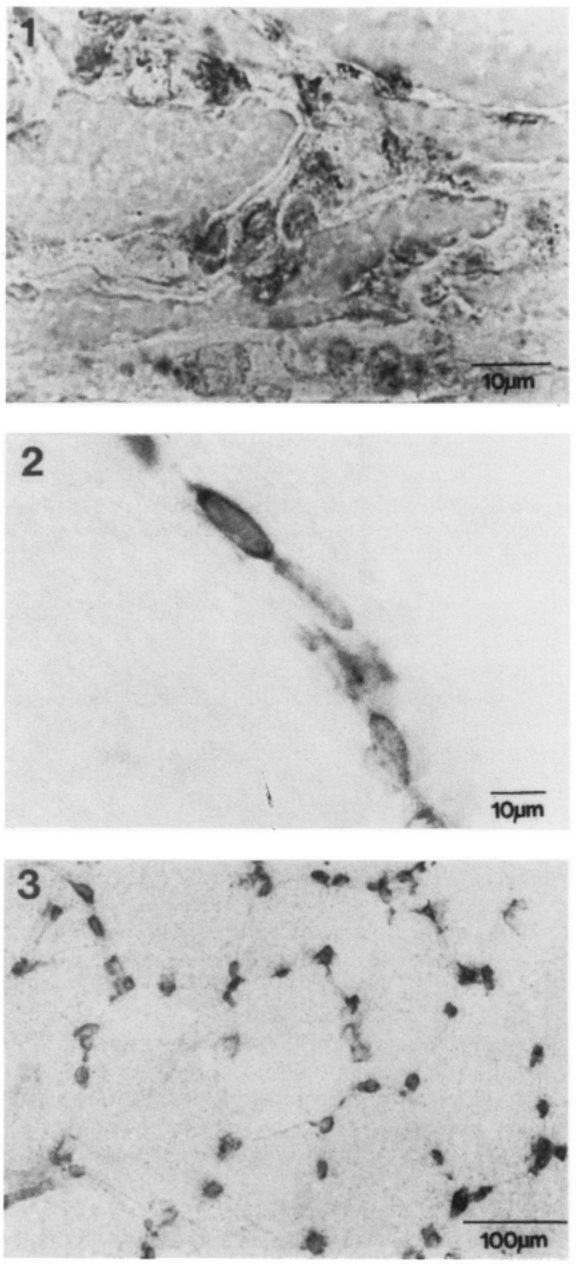

Figure 3. Localization of cathepsin L in the phagocytes near/in muscle fibers in the white muscle having very high catheptic activity. The autolytic activity was determined to be 89 units/g of tissue. A longitudinal section (2) and a cross section (3) of the muscle were stained with anticathepsin L. Strongly positive reactions for anticathepsin L were seen around necrotic muscle fibers (1).

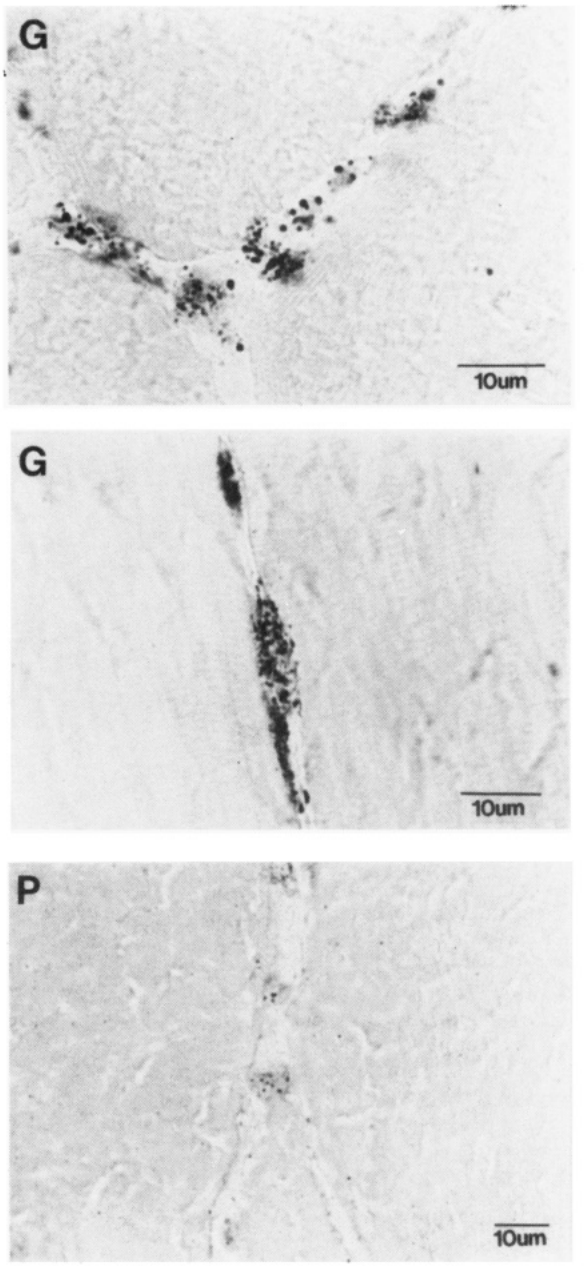

Figure 4. Localization of phagocytes in the white muscle of chum salmon. The phagocytes were stained according to the Giemsa stain method (G) and the peroxidase stain method (P).

Macrophage-like phagocyte has been reported to relate to intra- and extracellular protein breakdown in the normal functioning and pathological states of muscle tissues (Baici and Knopfel, 1986; Holtzman, 1989; Ishiura et al., 1983; Kominami et al., 1984). It was suggested by these studies that cathepsins B and L in phagocyte may take part in the protein breakdown in animal tissues and that phagocyte degrades connective tissue proteins such as collagen and elastin as well. In particular, cathepsin L is capable of hydrolyzing many different kinds of proteins including muscle structural proteins (Barrett and Kirschke, 1987; Yamashita and Konagaya, 1990d). In support of these considerations, the present finding offers corroborating evidence for participation of the phagocytes rich in cathepsins in the destruction of muscular structure in vivo.

From the food–chemical aspect of fish meat, there is a general postulation that the post-mortem autolysis of the muscles of fish and domestic animals as well is due to proteolysis of muscle structural proteins (Brown, 1986; Etherington, 1987; Greaser, 1986; Koohmaraie et al., 1988; Penny, 1980; Seki and Watanabe, 1988). Although many investigators have ambiguously thought possible the participation of muscle proteases such as cathepsins (Okitani et al., 1980) and calcium-dependent proteases (Koohmaraie et al., 1988; Penny, 1980; Seki and Watanabe, 1982) in the muscle softening, the cellular mechanism of the autolysis has not always been explained well by proteolysis theory. Our present observation suggests that cathepsin L in phagocytes appearing in the white muscle might participate in the extensive muscle softening during postmortem storage of the chum salmon. In previous studies (Konagaya, 1982; Yamashita and Konagaya, 1990a), cathepsin L was considered to be the most probable enzyme responsible for the extensive muscle autolysis which is observed in the catch of mature salmon. As this enzyme was present in phagocytes near the muscle fibers, the extensive softening will probably be caused by the pro-

teolysis of muscle structural proteins by cathepsin L liberated from the phagocytes present near/in muscle fibers. For in vivo or post-mortem activities of cathepsins, especially cathepsin L, it might be necessary for the enzyme to be secreted from living phagocytes or liberated from the phagocytes by disruption, for example, by freezing, respectively. The present study proposed an important concept that the nonmuscle cell, the macrophage-like phagocyte, is closely related to the muscle softening during the storage of salmon caught during spawning migration.

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