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Purification and characterization of a fish granzymeA involved in cell-mediated immunity

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ABSTRACT

Granzymes are serine proteases involved in the induction of cell death against non-self cells. The enzymes differ in their primary substrate specificity and have one of four hydrolysis activities: tryptase, Asp-ase, Met-ase and chymase. Although granzyme genes have been isolated from several fishes, evidence for their involvement in cytotoxicity has not yet been reported. In the present study, we attempted to purify and characterize a fish granzyme involved in cytotoxicity using ginbuna crucian carp. The cytotoxicity of leukocytes was significantly inhibited by the serine protease inhibitor "3, 4-dichloroisocoumarin". In addition, we found that granzymeA-like activity (hydrolysis of Z-GPR-MCA) was inhibited by the same inhibitor and significantly enhanced by allo-antigen stimulation *in vivo*. Proteins from leukocyte extracts were subjected to two steps of chromatographic purification using benzamidine-Sepharose and SP-Sepharose. The molecular weight of the purified enzyme was estimated to be 26,900 Da by SDS-PAGE analysis. The purified enzyme displayed a K_m of 220 μM, a K_{cat} of 21.7 sec^{-1} and a K_{cat}/K_m of 98,796 sec^{-1} M^{-1} with an optimal pH of 9.5 for the Z-GPR-MCA substrate. The protease was totally inhibited by serine protease inhibitors and showed granzymeA-like substrate specificity. Therefore, we conclude that the purified enzyme belongs to the mammalian granzymeA (EC 3.4.21.78) and appears to be involved in cytotoxicity in fish.

1. Introduction

Cell-mediated immunity plays crucial roles in the immune response against non-self antigens such as allografts, tumors and virus-infected cells. Non-self antigens can be degraded by the activities of specific and non-specific cytotoxic effector cells: cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. In ginbuna crucian carp (*Carassius auratus langsdorfii*), CTLs have been identified as CD8α$^+$ T cells possessing alloantigen- or virus-specific cytotoxicity (Somamoto et al., 2013; Toda et al., 2009). Non-specific cytotoxic cells (NCCs) and NK-like cells, both of which possess NK-like activity, have been reported in several fish species (reviewed in Fischer et al., 2013; Nakanishi et al., 2011).

Mechanisms of cell death induced by effector cells have been well characterized in mammals. After effector cells recognize non-self antigen, cytotoxic granules that contain several cell death molecules, e.g. the pore-forming protein "perforin" or granule-associated protease "granzymes", are secreted (Peters et al., 1991). Secreted perforin disrupts the cell membrane of target cells and results in granzyme entry into the cytoplasm (Trapani and Smyth, 2002). In the cytoplasm, granzymes hydrolyze several apoptotic molecules and activate additional apoptotic signals.

Granzymes are serine proteases classified based on their substrate specificities using synthetic substrates, and four different enzymatic activities have been reported: tryptase (cleaving after Arg or Lys), Asp-ase (cleaving after Asp), chymase (cleaving after Phe, Trp or Tyr) and Met-ase (cleaving after Met) (Kam et al., 2000). More than 10 granzymes have been reported, however only two (granzymeA and granzymeB) have been clearly defined with respect to enzymatic activity and function in the cell death pathway. GranzymeA and granzymeB are the most abundant granzymes and have been purified from cytoplasmic granules of human CTL (Krahenbuhl et al., 1988). These granzymes have clearly

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different substrate specificities and roles, e.g. granzymeB hydrolyzes caspase3 and induces caspase-dependent apoptosis (Andrade et al., 1998), while granzymeA hydrolyzes SET, HMG2 and Ape1, and induces activation of NM23-H1 DNase, which results in caspase-independent apoptosis (Chowdhury et al., 2006).

In fish, the perforin-granzyme pathway is assumed to play a major role in cell death induced by cytotoxic cells. It has been reported that cytotoxicity of alloantigen specific $CD8^+$ T lymphocytes is suppressed by the inhibition of perforin and/or a granzymeB-like protease (Toda et al., 2011a, 2011b). Although granzyme and perforin genes have been isolated in several fish species (Matsuura et al., 2014; Nakanishi et al., 2011), their function and mechanisms in cytotoxicity have not been characterized. For characterization, a biochemical approach at the protein level is required because it is difficult to predict their activities and roles from only genetic information.

In the present study, we characterize a fish granzymeA involved in leukocyte cytotoxic activity. We demonstrate that a serine protease was involved in cytotoxicity of allo-stimulated leukocytes against allogeneic cells, and that granzymeA-like activity was enhanced by allo-stimulation. Assays with purified enzyme revealed that it is a trypsin-like serine protease that can hydrolyze substrates containing either Arg or Lys amino acid residues at the P1 position. In addition, the substrate specificity was most similar to that of mammalian granzymeA (EC 3.4.21.78). These results suggest that the enzyme is a fish homolog of mammalian granzymeA involved in leukocyte cytotoxicity. This is the first report on the purification and characterization of an endogenous granzyme in fish.

2. Material and methods

2.1. Fish

Two isogeneic clones of ginbuna crucian carp from Lake Suwa (S3N clone) and the island of Okushiri (OB1 clone) were used. Fish, weighing 20–25 g, were maintained in 800 L tanks with running water at a temperature of 25 ± 1 °C and fed twice daily with commercial pellets.

2.2. Cell lines

The Ginbuna crucian carp cell line CFS has been established by Hasegawa et al. (1997) from fin tissue of an S3N clonal ginbuna. The cells were maintained in Leibovitz's L-15 medium (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc. 10% FBS-L-15) at 30 °C. The cells were passed when confluency was reached, every 2–3 days.

2.3. Allogeneic sensitization

Allogeneic sensitization of OB1 clone fish was performed as described by Matsuura et al. (2014). Briefly, sensitization was achieved by allogeneic scale grafting, followed by two injections with allogeneic cells at 7-day intervals. Scales from an S3N clone fish and CFS cells were used as allogeneic antigen. Seven days after the final sensitization, the fish were used for preparations of leukocytes.

2.4. Preparation of leukocytes

The fish were anesthetized with 50 mg/L benzocaine and bled from the caudal vessels. They were then deeply anesthetized with 50 mg/L benzocaine and their spinal cords were severed for euthanasia. Kidney cells were disaggregated by pressing through a 100-gauge mesh stainless steel sieve in Hank's Balanced Salt Solution (Nissui Pharmaceutical co., Tokyo, Japan) supplemented with 0.5% heat-inactivated FBS (0.5% FBS-HBSS). The kidney cells were washed twice by centrifugation at 500×*g* for 5 min at 4 °C with 0.5% FBS-HBSS. After washing, 2 mL of distilled water was added to the kidney cells to lyse erythrocytes. The cells were then washed three times with 0.5% FBS-HBSS by centrifugation at 500×*g* for 5 min at 4 °C. For enzyme assays, the leukocytes were washed three times with phosphate-buffered saline (PBS) and resuspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.2, 0.5% NP-40, 1 M NaCl). The lysates were held at −80 °C until use. After disrupting by passage through a 27-gauge needle attached to a 1 mL syringe (Terumo, Tokyo, Japan), a supernatant of the lysate was harvested by centrifugation at 16,000×*g* for 60 min.

2.5. Cytotoxicity assay

Cytotoxicity was measured using the method of Somamoto et al. (2002, 2006); with slight modifications. Target cells (CFS cells) were seeded in 96-well, flat bottom, half area plate wells (Greiner bio-one, Monroe, NC) at 2×10^3 cells/well in 100 μL of 10% FBS-L-15 and allowed to adhere to the wells for 6 h. The medium was then replaced by 100 μl of 10% FBS-Opti-MEM containing 2 μCi of $Na_2^{51}CrO_4$ (Muromachi Yakuhin co., Tokyo, Japan) and incubated overnight at 27 °C, 5% CO_2 to label the target CFS cells. After incubation, cells were washed four times with 10% FBS-Opti-MEM. Effector cells, prepared as in Section 2.3 above were resuspended in 10% FBS-Opti-MEM and incubated 6 h at 30 °C in 5% CO_2. After incubation, cells were resuspended in 10% FBS-Opti-MEM with 1 μM, 10 μM or 100 μM of each protease inhibitor (3, 4-dichloroisocoumarin (DCI, Sigma-Aldrich, St. Louis, MO) or E-64d (Peptide Institute, Inc., Osaka, Japan)) and incubated 3 h at 30 °C. Cells were then resuspended in the same medium with DCI or E-64d and mixed with ^{51}Cr labeled target cells (100 μL per each well) for 8 h at 27 °C in 5% CO_2. After incubation, supernatants were harvested and radioactivity was measured using an AccuFLEX γ ARC-7000 (Hitachi Aloka Medical, Tokyo, Japan). The radioactivity of the supernatants from the target cells without effector cells and from target cells lysed with detergent (10% SDS, Sigma-Aldrich), served as spontaneous and maximum release controls, respectively. Percent cytotoxicity was calculated as follows:

$$\frac{(\text{Test release}) - (\text{Spontaneous release})}{(\text{Maximum release}) - (\text{Spontaneous release})} \times 100$$

2.6. Enzyme assays

The following fluorogenic peptide substrates were used to measure protease activity: acetyl-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid α-4-methylcoumaryl-7-amide (Ac-IETD-MCA), benzoyl-L-arginine 4-methyl-coumaryl-7-amide (Bz-R-MCA), benzyloxycarbonylglycil-L-prolyl-L-arginine 4-methylcoumaryl-7-amide (Z-GPR-MCA), L-methionine 4-methyl-coumaryl-7-amide (M-MCA), and succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methyl-coumaryl-7-amide (Suc-AAPF -MCA), L-lysine 4-methylcoumaryl-7-amide (K-MCA), benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Z-FR-MCA), t-butyloxycarbonyl-L-leucylglycyl-L-arginine 4-methylcoumaryl-7-amide (Boc-LGR-MCA), t-butyloxycarbonylglycyl-L-lysyl-L-arginine 4-methylcoumaryl-7-amide (Boc-GKR-MCA), t-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methylcoumaryl-7-amide (Boc-VLK-MCA), succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc-GP-MCA), L-alanyl-L-alanyl-L-phenylalanine 4-methylcoumaryl-7-amide (AAF-MCA), succinyl-L-alanyl-L-prolyl-L-alanine 4-