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Development, characterization, and technical applications of a fish lysozyme-specific monoclonal antibody (mAb M24-2)

Marlee B. Marsh, Charles D. Rice *

Department of Biological Sciences, Clemson University, 132 Long Hall, Clemson, SC 29634, USA

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ABSTRACT

Lysozyme is one of several humoral and cellular factors associated with front line, innate immunity in all vertebrates. Historically, circulating lysozyme has been quantified in teleosts by measuring enzymatic activity against heat-killed *Mycococcus lysodieticus* using whole serum or plasma at a low pH. However, the amount of serum or plasma required for standard lysozyme activity exceeds that which can be easily acquired from small fish, thus making lysozyme a difficult endpoint to measure in limited sample volumes. Moreover, while circulating lysozyme is considered to be an indicator of proinflammatory phagocyte activity, the cellular source of this protein is not easily detected in fish. While several antibodies against lysozyme are commercially available for use in higher vertebrates, neither reacts with lysozyme in fish. In this study, a monoclonal antibody (mAb) for detecting and quantifying lysozyme was developed from mummichog, *Fundulus heteroclitus*, myeloid cells that also recognizes hen egg lysozyme (HEL), then tested for cross-reactivity in different species of teleosts. A single protein of ≈14–15 kDa mass was identified by the mAb in fish cell lysates and plasma samples, as well as denatured HEL. Total circulating lysozyme protein was compared to lysozyme activity using standard ELISA procedures and was found to correlate with enzymatic activity. Using mAb M24-2, intracellular lysozyme protein was detected in formalin-fixed and permeabilized lymphoid cells adhered to glass cover slips. Moreover, mAb M24-2 localizes lysozyme to myeloid cells. Finally, it was demonstrated that mAb M24-2 is suitable for immunohistochemistry in that lysozyme could be detected in plastic-embedded lymphoid tissues.

1. Introduction

Lysozyme is one of several anti-microbial proteins associated with front line, innate immunity in all vertebrates. This enzyme (muramidase) breaks the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of gram positive bacteria, and in association with complement components gram negative bacteria may be affected as well [1–3]. In higher vertebrates, lysozyme is an abundant component of primary and secondary granules of granulocytes [4], is secreted by macrophages [5–7], and circulating enzymatic activity is a biomarker for systemic inflammation [8]. Recent studies show that lysozyme has anti-oxidant properties [9], while others suggest it enhances the effects of complement [10] and may act as an opsonin to enhance phagocytosis by neutrophils and macrophages [11,12].

In fish, lysozyme genes are expressed in cells of myeloid origin [13,14] and very early in development [14], while enzymatic activity of the protein can be found in mucus, serum, and eggs [15–18]. There are also seasonal variations in circulating lysozyme activity [19]. Lysozymes of vertebrates are classified as either c-type or g-type, as both types are expressed in all vertebrates, with several

* Corresponding author. Tel.: +1 864 656 0449; fax: +1 864 656 0435.
E-mail address: cdrice@clemson.edu (C.D. Rice).

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subtypes of each being found among the taxa [20]. The relative contribution of each lysozyme type to overall enzymatic activity is unclear at this point, but gene expression profiles seem to vary among tissues and between species in response to pathogenic challenge [20].

Virtually all studies to date suggest that monocytes, macrophages and granulocytes are the main source of lysozyme. For example, exposure to β-glucan and lipopolysaccharide (LPS) increase lysozyme gene expression and enzyme activity in Atlantic salmon, *Salmo salar* L. anterior kidney macrophages [3]. The kinetics of lysozyme gene expression and enzyme activity in this study were both time and dose dependent, with LPS being a stronger inducer. Other studies show that immunization of adult female fish with potential bacterial pathogens leads to increased circulating lysozyme, which may be passed to the eggs after spawning [18]. Furthermore, exposure to chemical contaminants, including mercury, selenium, copper, herbicides, and co-planar PCBs modulate circulating lysozyme activity in fish [21–24].

Lysozyme activity in a biological sample can be quantified using simple techniques [25,26]. Typically, a volume of fish serum, plasma, mucus, cell lysate, or egg yolk is mixed with a suspension of heat-killed *Mycococcus lysodieticus* at an acidic pH. The optical density of the solution is then measured in the beginning and at a later time point for spectrophotometric analysis, or 18–24 h later for agar-based systems that quantify a zone of lysis. The rate of decrease in optical density or diameter of the zone of lysis is compared to a known lysozyme standard such as hen egg lysozyme (HEL), and enzymatic activity is calculated as units of HEL activity [25] or HEL equivalents [27].

The primary drawback for measuring lysozyme activity is that a minimum of 20–40 μl of sample is required for standard microtiter assays; a volume not readily available from small fish commonly used in laboratory studies. Therefore, an alternative means for quantifying circulating lysozyme is needed. The development of a lysozyme-specific antibody that cross reacts with a variety of fish species would allow researchers to quantify this important marker of myeloid cells and innate immune responses in very small amounts of material. To date, several antibodies generated against chicken (HEL) and human lysozyme are commercially available, and at least one generated against human urine lysozyme is cross-reactive among different species of higher vertebrates, including HEL (mAb BGN/06/961, cat. # ab36362—AbCam, Cambridge, MA, USA). To our knowledge, however, neither of these commercially available antibodies recognize lysozyme protein(s) in fish.

The work herein describes the development and characterization of a mAb for detecting, localizing, and quantifying lysozyme in fish as an indicator of innate immune status. Fish innate immune responses are routinely evaluated as indicators of immune function and status following exposure to pathogens, biological response modifiers, immunotoxicants, and nutritional regimes [28–31]. This particular study used the mummichog, *Fundulus heteroclitus*, a small estuarine minnow found from Newfoundland Canada to northeast Florida, USA. Mummichogs are routinely used in studies of developmental biology, environmental toxicology, and ecosystem health; and because it adapts well to laboratory settings.

2. Materials and methods

2.1. Fish and tissues

All animal studies were conducted within protocols approved by Clemson University's IACUC. Adult mummichogs, also known as the estuarine or marsh killifish, *F. heteroclitus* (8–12 g), were collected in the late summer with baited minnow traps at the Belle Baruch Marine Lab, Georgetown, SC, USA; which is located on a relatively pristine segment of the North-Inlet Winyah Bay National Estuarine Research Reserve. Fish without visible signs of trauma or disease were transported in aerated coolers to laboratory housing facilities at Clemson University and transferred to 100 l tanks containing aerated 15 parts per thousand artificial sea water (Instant Ocean) and maintained at 27 °C. After 7 days of acclimation to laboratory conditions, 100 fish of both genders were sacrificed by lethal anesthesia with 2 g/L MS-222 in ambient artificial seawater. Fish were bled from the caudal vasculature using a single-edged razor blade and capillary action within heparinized hematocrit tubes. The hematocrit tubes were centrifuged, after which the plasma from individuals was collected, pooled, and stored at 4 °C after addition of sodium azide (0.05%, w/v) until further analysis. Anterior kidney and spleens were collected, pushed through 80× mesh screens in Hanks balanced salt solution (166 mM NaCl, 6.5 mM KCl, 6.8 mM glucose, 0.05 mM KH_2PO_4, 0.07 mM Na_2HPO_4, pH 7.2), hereafter referred to as HBSS. Cells were centrifuged over Percoll (Sigma Chemical Company, St. Louis, MO, USA) gradients of 1.050 and 1.080 g ml^{-1} as previously described for gulf killifish, *Fundulus grandis* [32], a closely related species of the mummichog. This isolation procedure concentrates macrophages and granulocytes. Cells were collected, counted, and pelleted by centrifugation to yield 5×10^6 cells per pellet, and used directly for immunizing mice, or frozen at −80 °C as dry pellets for booster immunizations. Additional cells were frozen as a source of intracellular proteins.

2.2. Monoclonal antibody production

Anterior kidney leukocyte pellets were suspended in HBSS and mixed with TiterMax Gold adjuvant (TiterMax USA, Inc., Norcross, GA, USA). Six week-old-female RBF/dnj mice (Jackson Labs, Bar Harbor, ME, USA) were immunized subcutaneously and intraperitoneally with 100 μL of the preparation at each site. Subsequent booster immunizations with thawed cell pellets in HBSS were administered without adjuvant. Booster schedules, fusion procedures, and hybridoma screening techniques followed those previously described by Rice et al. [33]. To screen primary hybridomas for antibodies recognizing lysozyme-like protein, frozen cell pellets were added to ice-cold lysis buffer (250 mM NaCl, 25 mM Tris–HCl, and 1 mM PMSF, pH 8, with 1% NP-40) containing HALT protease-inhibitor