Responses of Atlantic cod *Gadus morhua* head kidney leukocytes to phytase produced by gastrointestinal-derived bacteria

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Abstract This study identified phytase-producing bacteria that were previously isolated from the gastrointestinal tract of Atlantic cod, *Gadus morhua* and determined its effect on head kidney leukocytes. Out of the 216 bacterial strains tested, the two phytase producers were identified as *Pseudomonas* sp. and *Psychrobacter* sp. based on their 16S rDNA sequence. Crude phytase from these two bacterial strains was produced employing the shake flask method. Even though the total protein of the crude phytase was not significantly different for the two bacteria, the phytase activity of the crude enzyme produced by *Pseudomonas* sp. (97.1 ± 16.7 U) was significantly higher than that of the enzyme from *Psychrobacter* sp. (75.9 ± 2.4 U). When cod head kidney leukocytes were incubated with the crude phytase (50 µg ml^{-1}), it resulted in enhanced cell proliferation, higher myeloperoxidase, and acid phosphatase activities. Extracellular responses—respiratory burst activity and hydrogen peroxide production were not enhanced by the crude enzyme. As a consequence, the growth of two pathogenic bacteria *Aeromonas salmonicida* and *Vibrio anguillarum* was not suppressed by the supernatants obtained from head kidney leukocytes incubated with the crude bacterial phytase. Thus, the enzyme from phytase-producing intestinal bacteria of Atlantic cod can stimulate intracellular head kidney leukocyte activities but not the production of extracellular substances that are involved in antibacterial response. These have implications on the potential use of bacterial phytase as feed supplement to boost cellular immune response of the fish and could be employed as a health management strategy in culture systems.

Keywords Phytase · Cellular response ·
HK leukocytes · Atlantic cod · Gastrointestinal tract

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Introduction

Phytases (myo-inositol hexaphosphohydrolases; E.C. 3.1.3.8) are histidine acid phosphatases which catalyze the hydrolysis of phytic acid (myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate) which is the predominant form of phosphorus in cereal grains, oilseeds, and legumes (Yoon et al. 1996; Vats and Banerjee 2005). This enzyme is widespread in nature because it is found in animals, plants, and microorganisms (Cao et al. 2007). Sustainable and environmentally friendly aquafeeds rely on plant-based protein sources. However, the phytate-bound phosphorus in plant ingredients are not available to fish that are monogastric or agastric, and hence phytase supplementation is necessary to digest phytate and release phosphorus

(Cho and Bureau 2001). Microbial phytases are the most popular among phytases because they have a higher spectrum of enzymatic activity.

The resident microbiota of the fish gut is recognized as producers of enzymes that are actively involved in digestion (Saha et al. 2006; Mondal et al. 2008). Among the enzyme-producing microorganisms that are not well studied is the group that can produce the enzyme phytase. Research on this class of microorganism of aquatic origin is limited, although they have been isolated from the gut of some fishes (Hirimuthugoda et al. 2006; Roy et al. 2008). To our knowledge, no reports have documented the presence of the phytase-producing bacteria in the gastrointestinal tract of Atlantic cod. It is also acknowledged that a single phytase may never be able to meet the diverse needs—neither from the physiological point of the animal nor from its commercial and environmental applications (Roy et al. 2008). Therefore, isolating new strains of phytase-producing bacteria, particularly from among the resident microbiota of the host is very interesting.

The enzyme industry today is constantly searching for new areas of application (Choct 2006). Most research on phytase are directed toward determining its effect on bioavailability of phosphorous, proteins and other nutrients, and the growth performance of fish (Cao et al. 2007). Investigations on phytases should not be limited to its nutritional application. Exploration of the areas which are of great interest and importance, such as its possible role in the immune system, would reveal whether such an attribute exists in fish.

In this study, bacterial strains isolated from the gastrointestinal tract of Atlantic cod (Gadus morhua L.) were screened for phytase production and characterized at the molecular level. Further, the cellular immune responses and antibacterial activity of cod head kidney leukocytes incubated with phytase produced by the identified phytase-producing bacteria were determined.

Materials and methods

Fish

Apparently healthy Atlantic cod weighing about 2 kg were fished from the coastal waters off Bodø and transferred to the Mørkvedbukta Research Station at Bodø University College, Norway. The fish were sampled immediately or starved for 3 weeks prior to sampling. This study was part of an earlier study (Dhanasiri 2007). Fish handling and experimental procedures were approved by the National Animal Research Authority (FDU).

Screening for phytase production by bacteria isolated from the gastrointestinal tract of Atlantic cod

Bacteria isolated from the gastrointestinal tract of Atlantic cod were screened for their phytase producing ability. The bacterial strains were grown on modified phytase screening medium (MPSM) agar (Roy et al. 2008) supplemented with 1.5% sodium chloride (NaCl; w/v). The plates were incubated for 48 h at 15°C. The bacterial growth in this specific medium indicates the phytase producing capacity of that particular bacterium.

Molecular characterization of the isolated phytase-producing bacteria

The bacterial strains that were positive for phytase production were subjected to molecular characterization of their 16S rDNA. Bacterial genomic DNA was extracted using the previously described method (Caipang et al. 2003), and the 16S rDNA was amplified using the eubacterial universal primers (Bianciotto et al. 2003). The PCR products were sequenced using Big Dye Terminator ver 3.1 (Applied Biosystems, USA), and comparative sequence analysis was carried out on the sequences available in NCBI (National Center for Biotechnology Information) employing nucleotide BLAST (Basic Local Alignment Search Tool).

Preparation of crude phytase and determination of phytase activity

The identified phytase producing bacteria were used for preparing the crude enzyme. The bacterial strains were cultured in tryptic soy broth supplemented with 1.5% NaCl for 24 h at 15°C. About 5 ml of the seed culture were added to 250 ml of liquid media (MPSM without agar) and incubated with constant shaking for 72 h at 15°C. The cell-free supernatant was prepared

by centrifugation at 10,000 g for 10 min at 4°C and was used for enzyme assay and for in vitro examination of its effect on immune cells of Atlantic cod. Prior to enzyme assay, the total protein content of the crude phytase was determined using a protein assay kit (Molecular Probes, Eugene, OR, U.S.A.) according to the manufacturer's protocol. The phytase assay was performed with phytic acid as a substrate (Greiner and Farouk 2007) and the enzyme activity was determined spectrophotometrically at 415 nm. One phytase unit (U) was defined as the amount of enzyme per mL of culture filtrate that released 1 μg of inorganic phosphate.

Isolation of head kidney leukocytes and incubation with crude phytase of the selected bacteria

Head kidney (HK) leukocytes (predominantly macrophages) from Atlantic cod were prepared as described by Chung and Secombes (1988) with some modifications. In brief, head kidney pieces were mashed in a 100-μm nylon cell strainer (Bioscience Discovery Labware, Two Oak Park, Bedford, USA) with a stepwise addition of L-15 medium (with 50 U/ml penicillin [PN], 50 μg/ml streptomycin [SP], 2% fetal bovine serum [FBS], and 10 U/ml heparin). The cell suspension was centrifuged at 3,000 rpm for 5 min at 4°C. Thereafter, the cells were washed two times with L 15 medium. The washed cells were loaded slowly in a gradient containing 34% and 51% Percoll solutions and were centrifuged at 1,800 rpm for 30 min at 4°C. The cells banding at the 34–51% Percoll interface (leukocytes layer) were transferred into a 15-ml sterile tube and were washed three times with L-15 medium. Cell number was quantified using a flow cytometer (Cell Lab Quanta MPL, Bechman Coulter Ireland Inc., Galway, Ireland). Cell viability was determined by microscopy by trypan blue exclusion test.

Two milliliters (2 ml) of the suspension containing approximately 10^7 cells ml^{-1} was placed in each well of the 12-well plate and incubated for 6 h at 12°C for cell attachment. Later, the cell culture medium was aspirated to remove cell debris and unattached cells, washed with 1× sterile phosphate buffered saline (PBS) and replaced with fresh culture medium just prior to their use. The crude phytase diluted in sterile PBS were added to each well at a concentration of 50 μg ml^{-1}. The control cells were treated only with 1× PBS.

In vitro studies with Atlantic cod head kidney leukocytes

The cellular proliferation was measured using the 3-(4,5 dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT; Sigma, Steinheim, Germany) assay (Caipang et al. 2005). HK leukocytes (10^6 cells ml^{-1}) in a 96-well plate were incubated with the crude phytase for 3 and 24 h and added with 50 μl of fresh medium containing 0.5 mg of MTT ml^{-1}. The plate was wrapped in aluminum foil and further incubated for 1 h at 15°C. The crystals that formed in each well were dissolved using 150 μl of a solution containing 125 μl of dimethylsulfoxide (DMSO; Sigma) and 25 μl of glycine buffer (0.1 M glycine, 0.1 M NaCl [pH 10.5]), and incubated for 10 min at 15°C. Absorbance was read at 570 nm using a microplate reader (Fluostar Optima; BMG Labtech GmbH, Offenburg, Germany).

The myeloperoxidase activity was determined by lysing the leukocytes with 60 μl of 0.02% cetyltrimethylammonium bromide (CTAB; Sigma) and assayed according to the procedures of Quade and Roth (1997) with some modifications. The lysed cells were supplemented with 35 μl of 20 mM 3,3′,5,5′-tetramethyl benzidine hydrochloride (Sigma) and 35 μl of 5 mM H_2O_2. After a two-minute incubation, 35 μl of 4 M sulphuric acid was added to stop the reaction and the spectrophotometric measurement was taken at 450 nm using a microplate reader (Fluostar Optima).

Acid phosphatase (AP) activity was assayed using the method of Meng et al. (2003). HK leukocytes were lysed with 50 μl of 0.1% Triton X-100 (Sigma) at 4°C for 30 min, and then incubated at 37°C for 30 min to inactivate glucose-6-P. This was followed by the addition of 100 μl of 12 mM p-nitrophenyl phosphate (Sigma) in 0.2 M acetate buffer (pH 5.0). After further incubation at 15°C for 1 h, the reaction was stopped by the addition of 50 μl of ice-cold 0.01 M EDTA in 0.1 M NaOH, and absorbance was read immediately at 410 nm using a microplate reader (Fluostar Optima).

The superoxide anion ($O_2^{\bullet-}$) production in leukocytes was assayed using the nitroblue tetrazolium (NBT; Chung and Secombes 1988). One hundred

microliters of 0.4 mg ml^{-1} NBT (Sigma) in L-15 medium were added to the wells of a 96-well plate holding HK leukocytes (10^6 cells ml^{-1}). The cells were further incubated at 12°C for 1 h, and the reaction was stopped by fixing the cells with 100% methanol for 2-3 min. The cells were washed with 70% methanol, air-dried for 5 min, and the reduced formazan with leukocytes was solubilised in 120 μl of 2 M KOH and 140 μl of DMSO (Sigma). The optical density was determined at 630 nm using a microplate reader (Fluostar Optima).

The hydrogen peroxide (H_2O_2) production in HK cells was measured using the horseradish peroxidase (HRP)-dependent oxidation of phenol red with some modifications (Chung and Secombes 1988). In brief, the HK cells were covered with 10 μl of phenol red solution containing 140 mM NaCl, 10 mM potassium phosphate buffer (pH 7.0), 5.5 mM glucose, 0.2 g ml^{-1} phenol red, and 100 μg ml^{-1} horseradish peroxidase (HRP; Sigma), and incubated at 15°C for 1 h. The reaction was stopped by the addition of 10 μl of 1 M NaOH and then spectrophotometrically determined at 630 nm.

All the assays were performed at 3 and 24 h post-incubation (hpi) with the crude phytase, and the absorbance readings were corrected from the negative control, which consisted of the cell culture medium, assay reagents, and the crude phytase.

Bacterial proliferation

Proliferation of the bacterial pathogens in supernatants from primary HK leukocyte cultures incubated with crude phytase was determined using a co-incubation assay (Caipang et al. 2008). *Vibrio anguillarum* strain VI-F-258-3, which is a serotype

O2β and *Aeromonas salmonicida* strain NCIMB 1102 provided by the Institute of Marine Research, Bergen, Norway were used as target pathogens. Bacterial activity was measured by the addition of MTT (2 mg ml^{-1}) to the supernatant-bacteria solution after a 24-h incubation at 15°C. The MTT reduction was measured employing a microplate reader (Fluostar, Optima) at 630 nm, and the index of bacterial growth was determined following Budiño et al. (2006). Bacterial suspension inoculated in tryptic soy broth (TSB, Fluka Biochemika) served as medium control.

Statistical analysis

The data are represented as mean ± SD and analyzed using one-way analysis of variance (ANOVA) (Systat ver 8). If the observed differences were significant, then the *t*-test for independent samples was further used. Significance levels were set at $P < 0.05$.

Results

Identification of the phytase-producing bacteria

Two hundred and sixteen bacterial strains previously isolated from the intestinal tract of wild-caught Atlantic cod were screened for phytase activity. Two bacterial strains gave a positive result for phytase activity based on their growth in the modified phytase screening medium. The bacterial strains were identified as *Pseudomonas* sp. and *Psychrobacter* sp. since they had the closest homology to *Pseudomonas cf synxantha* and *Psychrobacter glacincola* respectively based on their 16S rDNA sequence (Table 1). The phytase activity of the crude enzyme produced

Table 1 Identity of the phytase-producing bacteria isolated from the gastrointestinal tract of Atlantic cod (*Gadus morhua*) and biochemical properties of the phytase produced

Characteristics	GP 21	GP 12
Gram staining	Negative	Negative
Nearest homology based on 16S rDNA	*Psychrobacter glacincola* (FJ449639.1)[1]	*Pseudomonas cf. synxantha* (AJ244725.1)[1]
Homology (%)	100	97
Phytase activity (U)	75.9 ± 2.4[a]	97.1 ± 16.7[b]
Crude phytase protein content (μg ml^{-1})	6110 ± 0.60	5530 ± 0.36

[1] Accession number of the nearest homology of the bacterial strain found in the NCBI BLAST: Nucleotide Sequence Database

[a,b] Indicates significant difference at $P < 0.05$

by *Pseudomonas* sp. was significantly higher compared to that of *Psychrobacter* sp. However, the total protein content of the crude phytase from both bacterial strains was not significantly different.

Leukocyte proliferation, myeloperoxidase, and acid phosphatase activities

Cellular responses were observed on incubation of HK leukocytes with crude phytase from the phytase-producing bacterial strains at 3 and 24 hpi. The crude phytase from the two bacteria elicited higher proliferation of HK leukocytes at 3 hpi (Fig. 1). However, at 24 hpi, only the enzyme from *Pseudomonas* sp. produced a significant difference relative to the control group.

Myeloperoxidase activity of the HK leukocytes incubated with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. was significantly higher than the control at 24 hpi (Fig. 2). In addition, acid phosphatase activity in HK leukocytes incubated with the phytase from both bacterial strains was significantly higher than the control group at 3 and 24 hpi (Fig. 3).

Respiratory burst activity and hydrogen peroxide production

Respiratory burst activity of the HK leukocytes incubated with crude phytase from both bacterial strains was not significantly different from the control at 3 hpi (Fig. 4). However, a significant decrease in respiratory burst activity was observed in cells
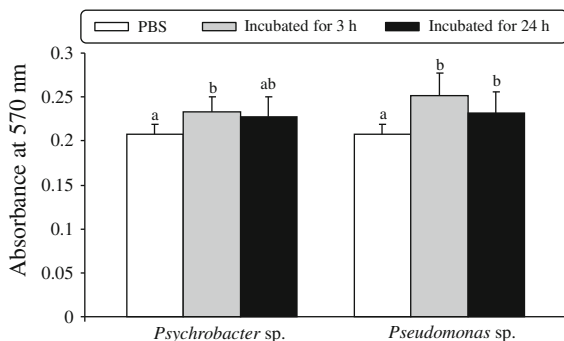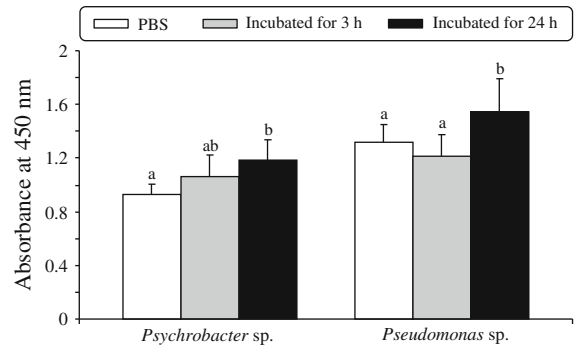
Fig. 2 Myeloperoxidase activity of head kidney leukocytes upon incubation with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. taken at 3 and 24 hpi. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD, $n = 8$
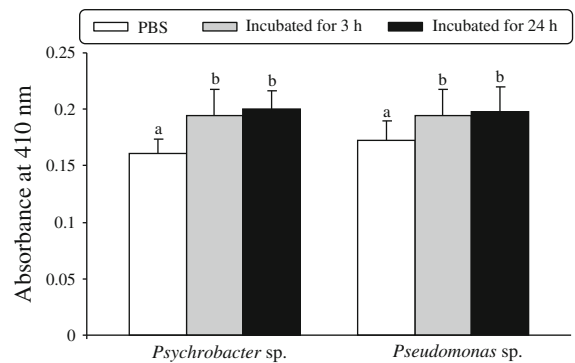
Fig. 3 Acid phosphatase activity of head kidney leukocytes on incubation with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. taken at 3 and 24 hpi. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD, $n = 8$

Fig. 1 Changes in the proliferation of the head kidney leukocytes on incubation with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. taken at 3 and 24 hpi. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD, $n = 8$
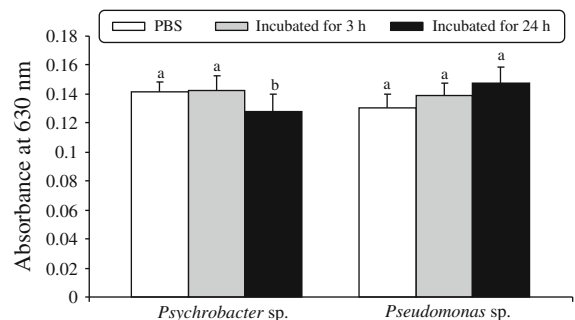
Fig. 4 Respiratory burst activity of head kidney leukocytes on incubation with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. taken at 3 and 24 hpi. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD, $n = 8$

incubated with the crude enzyme from *Psychrobacter* sp. at 24 hpi.

There was no significant difference in the hydrogen peroxide production by the HK leukocytes incubated with crude phytase from *Psychrobacter* sp. at 3 and 24 hpi (Fig. 5). However, hydrogen
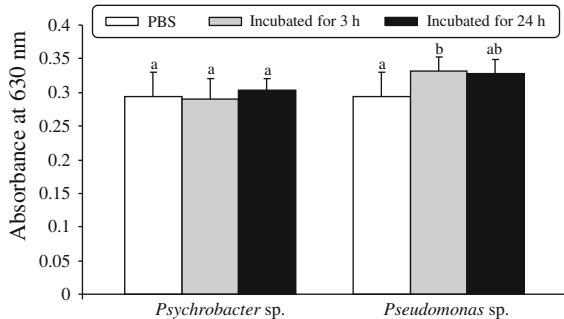

Fig. 5 Hydrogen peroxide production by the head kidney leukocytes upon incubation with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. taken at 3 and 24 hpi. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD, $n = 8$
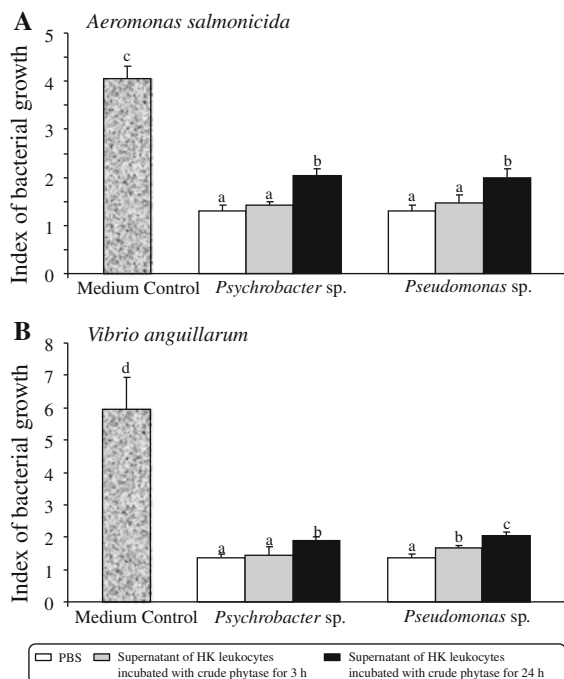

Fig. 6 Proliferation of bacterial pathogens in supernatants from head kidney leukocytes incubated with crude phytase from *Psychrobacter* sp. and *Pseudomonas* sp. for 3 and 24 h. **a** *Aeromonas hydrophila* and **b** *Vibrio anguillarum*. Column bars with different letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD, $n = 8$

peroxide production of the leukocytes exposed to *Pseudomonas* sp. crude enzyme was significantly higher at 3 hpi.

Proliferation of bacterial pathogens
in supernatants of HK leukocytes incubated
with the crude bacterial phytase

In comparison with the medium control (TSB), the proliferation of *A. salmonicida* (Fig. 6a) and *V. anguillarum* (Fig. 6b) was significantly reduced in supernatants derived from HK leukocytes that were pre-incubated with PBS and crude phytase of the bacterial isolates. However, when compared with supernatants derived from HK leukocytes incubated with PBS, the growth of the two bacterial pathogens significantly increased in supernatants of HK leukocytes that were pre-incubated with the crude phytase of both bacterial strains for 24 h.

Discussion

This study dealt with two relatively unexplored, yet interesting areas of phytase research—identifying the presence of phytase-producing bacteria in the fish gastrointestinal tract and understanding their influence on the fish immune system.

Fish either lack or have only low levels of phytase in their intestine (Roy et al. 2008) and, therefore, phytase needs to be supplemented into fish feeds (Cao et al. 2007). The benefits of phytase supplementation to plant-derived fish feed are well documented (Dalsgaard et al. 2009). Many studies have examined potential microorganisms for phytase production (Yoon et al. 1996; Nakamura et al. 2000; Sequeilha et al. 2000; Vats and Banerjee 2005; Huang et al. 2006; Greiner and Farouk 2007; Gulati et al. 2007), but our interest is to find native microorganisms of the fish gastrointestinal tract, which are capable of producing phytase, which we believe has great potential for aquaculture applications.

To our knowledge, this is the first article on the presence of phytase-producing bacteria in the intestinal tract of Atlantic cod. We identified two phytase-producing bacteria which we characterized molecularly as *Pseudomonas* sp. and *Psychrobacter* sp. and code named them as GP21 and GP12, respectively. The activity of the crude phytase from *Pseudomonas*

sp was significantly higher compared to that from *Psychrobacter* sp. Some members of the genus *Pseudomonas* have been identified as phytase-producers, and most of them are of soil origin (Richardson and Hadobas 1997; Park et al. 2002; Cho et al. 2003). These include *Pseudomonas syringae* (Cho et al. 2003; Cao et al. 2007), *Pseudomonas putida* (Richardson and Hadobas 1997), *Pseudomonas mendocina* (Richardson and Hadobas 1997), *Pseudomonas fragi* (In et al. 2004), and other species (Kim et al. 2002; Mukesh et al. 2004). The *Psychrobacter* sp. identified in this study is the first bacterial species isolated from the fish gastrointestinal tract that is reported as a phytase producer. Even though studies on phytase-producing microorganisms from aquatic animals or from the environment are very few, our observation opens up new areas of study. Hirimuthugoda et al. (2006) reported the presence of potential phytase-producing yeasts of marine origin. The marine yeast strain *Kodamea ohmeri* BG3 isolated from the gut of a marine fish (*Hexagrammes otakii*) was found to secrete a large amount of phytase into the medium (Li et al. 2008a), and a cost-effective production technique has been optimized using oats as medium (Li et al. 2008b). Recently, Roy et al. (2008) identified *Bacillus licheniformis* as a phytase-producing bacterium in the digestive tracts of some freshwater fish.

The phytase activity of the crude enzyme produced by the two bacterial strains was comparable to the phytase from several aquatic microorganisms. The crude enzyme produced had phytase activities of 75.9 U (GP21) and 97.1 U (GP12), respectively. These values were higher compared with the enzyme produced by phytase-producing microbiota of carp and tilapia that ranged from 1.03 to 1.87 U (Roy et al. 2008). An Antartic yeast strain, *Cryptococcus laurentii* had an activity of 72–82 U (Pavlova et al. 2008). A much higher phytase activity was observed from a marine yeast *K. ohmeri* BG3—941.2 U (Li et al. 2008c).

The effects of the crude phytase on the innate immune system of Atlantic cod were investigated using HK leukocytes. Five immune parameters were used to determine the cellular responses on incubation with the crude bacterial phytase. Even though the crude enzyme from the isolated strains activated the HK leukocytes, the responses were different. Both bacterial phytases induced leukocyte proliferation,

and this was significant at 3 hpi (Fig. 1). Our study showed a direct action of phytase to the cells. The presence of phytase could have triggered a series of cellular activities that resulted in proliferation, but additional studies need to support this speculation. In an indirect mechanism, specifically through absorption, an increased percentage of erythrocyte rosette-forming cells and erythrocyte-antibody complement cells were observed when chickens were fed with phytase-containing nutritionally marginal diets (Liu et al. 2008). The same study also showed that the percentages of $CD4^{+}CD8^{+}$ T lymphocyte subsets were increased by the addition of phytase into the diet. The hydrolysis of the phosphate group of the phytate molecule in the action of phytase yields lower molecular weight myoinositol phosphates, inositol and inorganic phosphates, in the gastrointestinal tract (Greiner et al. 2000). Liu et al. (2008) elucidated further that the lower inositol phosphates produced during phytate hydrolysis could induce factors that trigger the proliferation of the head kidney leukocytes. Even if different mechanisms were involved between these findings and the previous researches, the ability of phytase to influence cellular proliferation was a noteworthy finding.

Acid phosphatase (AP) is one of the important enzymes in the degradation of phagocytized pathogens, thereby preventing their growth and multiplication in the host (Attwood et al. 1996). On the contrary, myeloperoxidase (MPO) in the presence of halide ions and H_2O_2 can kill bacteria by halogenation of the bacterial cell walls as well as by the production of bactericidal hypohalite ions (Klebanoff and Clark 1978). Our study showed that incubation of cells with the crude phytase increased the activity of these two important enzymes involved in the intracellular responses. The increase in AP activity was significant for both the observation points (Fig. 3). This enzyme is localized within the lysosomes and is important in the intracellular digestion of phagocytized antigens (Meng et al. 2003). The MPO activity of the HK leukocytes was also enhanced, but a significant increase was observed only at 24 hpi suggesting a delayed response. This is due to the fact that leukocytes produce little, or insignificant amounts of myeloperoxidase (Alcorn et al. 2003).

Even though it was observed that crude bacterial phytase can activate HK leukocytes leading to its proliferation and significant increase in their MPO

and AP activities, minimal effects were observed in the respiratory burst activity and hydrogen peroxide production (Figs. 4, 5). The ability of cells to kill bacteria is dependent on the production of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, H_2O_2, and OH^- (Meng et al. 2003). The insignificant production of the two extracellular antibacterial substances resulted in the proliferation of bacterial pathogens in the supernatants from the HK leukocytes. The growth of the pathogenic bacteria, *A. salmonicida* and *V. anguillarum*, was not suppressed by the supernatants obtained from HK leukocytes on incubation with bacterial phytase (Fig. 6). This provides evidence that extracellular production of ROS is important for bacterial killing in Atlantic cod. The possible explanation for the pathogen proliferation upon phytase exposure of HK leucocytes is either that (1) the crude enzyme may have blocked the receptors of the cells thereby incapacitating their ability to produce substances that are responsible in inhibiting these pathogenic microorganisms or that (2) the dosage of the enzyme applied may have impaired the cellular responses of the leukocytes. It would be interesting to characterize the different leukocyte receptors in Atlantic cod that are responsible for pathogen recognition to better explain the activity of the bacterial phytase.

In conclusion, phytase-producing bacteria are present in the gastrointestinal tract of Atlantic cod, and this group of enzyme-producing microorganisms is of great interest and needs further research. The crude phytase produced by these bacteria stimulated head kidney leukocytes by enhancing cell proliferation, MPO and AP activities. Nevertheless, the respiratory burst activity and hydrogen peroxide production were not significantly upregulated, and the leukocytes might have been rendered incapable of preventing the growth of pathogenic bacteria. Thus, the crude phytase from the resident gastrointestinal bacterial strains of Atlantic cod could stimulate the intracellular, but not the extracellular responses of the HK leukocytes. Future studies will involve the biochemical characterization and purification of the phytase from these two bacterial strains and explore their immunomodulatory effects in fish. These may have significant impact on the development of feed supplements and health management in aquaculture systems.

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