



## Note

# Biochemical characterisation of lysozyme extracted from rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)

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## ABSTRACT

In the present study, lysozyme was extracted from spleen tissue of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) and partially purified by ammonium sulfate precipitation. Optimum pH and temperature as well as effects of different salt concentrations of NaCl, MgCl<sub>2</sub>, KCl and urea on enzyme activity were evaluated. The enzyme activity was assayed using a suspension of *Micrococcus lysodeikticus* as substrate. Results showed that lysozyme extracted from the spleen of *O. mykiss* has its optimum activity at high temperature (60°C) and low pH (5.8) conditions and the enzyme activity was found to vary with different salt concentrations which were found related to the environmental conditions of natural habitats of *O. mykiss*.

Keywords: Enzymology, Immune System, Lysozyme, Rainbow trout

The first line of defense against infectious pathogens is provided by the non-specific immune responses or innate immune system (Abdollahi *et al.*, 2016). The innate immune system is composed of two distinct parts: cellular and humoral. The mucus layer of fish is the first line of defense against pathogens (Gao, *et al.*, 2016). Fish mucus layer has several hydrolytic enzymes including lysozyme, alkaline-phosphatase, Cathepsin-B and proteases (Subramanian *et al.*, 2008). Mucus layer of skin which cover the scaled and non-scaled body parts plays its roles in defense by preventing pathogens from attachment, ion and osmoregulation and finally act as a reservoir of several innate immune factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, proteolytic enzymes and different antibacterial proteins and peptides (Modanloo *et al.*, 2017). It has been clearly demonstrated that fish mucus layer (on the skin, gill and in the intestine), is a key factor in immune responses (Ghalambor *et al.*, 2020).

Adrenal gland and spleen are the other two parts of innate immunity in teleost fish (Grove *et al.*, 2006). It has been previously documented that the fish adrenal gland and spleen are composed of immune cells with multiple sizes and roles, which are responsible in the secretion of humoral compounds including lysozyme and acid phosphatase (ACP) as important components of the innate immunity (Magnadottir, 2006). As mentioned above, one of the components of the humoral system is lysozyme which is known to have antibacterial activity by breaking down of the peptidoglycan bonds of the bacterial cell walls, especially in Gram-positive bacteria (Palaksha *et al.*,

2008). Lysozyme is found in invertebrates, vertebrates, plants and bacteria. Fish lysozyme is generally found in hematopoietic tissue of the head, kidney, spleen, skin, gill and digestive system. It has been demonstrated that, after incidents such as stress, injections of foreign materials or infections, the concentration of lysozyme increases in the fish body (Uribe *et al.*, 2011).

Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) belonging to the family Salmonidae is an economically and ecologically important fish species worldwide. This is one of the main aquacultured fish species and is spread in the vast majority of rivers and lakes all over the world. The aim of the present study was to evaluate the biochemical characteristics of lysozyme enzyme extracted from the spleen tissue of *O. mykiss*.

Fish were sampled from a fish farm at Dezful, Iran and 5 live adult *O. mykiss* (1-1.5 kg in body weight and 30-32 cm body length) were transferred to the laboratory and immediately submerged in a solution of clove oil as anesthetic and then dissected. Tissue samples of spleen, liver, gill and skin mucus were collected and after washing in physiological saline, the samples were stored in lysis buffer at -70°C (Bazrkar and Aghamaali, 2015).

One gram each of the tissue sample and 1 g of skin mucus in lysis buffer was sonicated using a sonicator (BANDELIN HD2200) and then shaken for 1h using Jeio Tech SK300. Subsequently, samples were centrifuged at 4°C at 5000 rpm using Hettich UNIVERSAL 320R and the supernatant was collected. Samples were then analysed by SDS-PAGE using BioRad-Mini PROTEAN

tetra system and based on the results obtained, spleen tissue was selected for continuing the study (Bazrkar and Aghamaali, 2015).

Spleen tissue (1 g) was kept in 0.2 M phosphate buffer (pH 6.2) and then for complete lysis and homogenisation of the cells, samples were sonicated using BANDELIN HD2200. Samples were then centrifuged at 4000 rpm at 4°C for 25 min using Hettich UNIVERSAL 320R. The supernatant (4500 µl) was transferred to a new tube and kept at -70°C for further evaluations (Bazrkar and Aghamaali, 2015). Ammonium sulfate (1.68 g) was added to 4500 µl supernatant and then centrifuged at 9000 rpm at 4°C for 15 min. The supernatant was removed and the precipitate was used for subsequent evaluations. Sodium phosphate buffer (0.2 M) was added to the precipitate and transferred to a dialysis bag with 2 l of 30 mM ammonium bicarbonate buffer and dialysed at 4°C for 36 h. The sample was then lyophilised with Christ Alpha 1-2LD plus and then 1 mg ml<sup>-1</sup> concentration of the sample was dissolved in 0.1 M phosphate buffer at pH 6.2 and then stored at -20°C as stock. Egg lysozyme (Sigma) was used as standard with a concentration of 1 mg ml<sup>-1</sup> in 0.1 M phosphate buffer (Bazrkar and Aghamaali, 2015).

To determine the effect of temperature on enzyme activity, *Micrococcus lysodeikticus* (Sigma) suspension was prepared by dissolving 0.0038 g of lyophilised powder of bacteria in 25 ml of sodium phosphate buffer (pH 6.2) and 2.9 ml each of the bacterial suspension was transferred to clean tubes. For each temperature, two tubes were used: standard tube containing egg lysozyme and the other one containing the sample. Each tube was examined at temperatures of 30, 40, 45, 50, 55, 60 and 80°C and incubated in ben-Mari (Membert WNB14) for 50 min. Enzyme kinetics was assayed using a spectrophotometer (Spectrum sp-uv200) at 450 nm for 3 min at intervals of 30 s (Bazrkar and Aghamaali, 2015).

Three different salts *viz.*, NaCl, MgCl<sub>2</sub> and KCl were used to analyse the effects of salt concentrations on enzyme activity. Multiple concentrations from each salt were prepared at 20, 40, 60, 80 and 100 mM. The bacterial suspension of *M. lysodeikticus* was prepared as described above and the suspension added to salt solutions and then optical absorption of each concentration was measured twice, first with standard (egg lysozyme) and second with the sample at 450 nm for 3 min at intervals of 30 s (Bazrkar and Aghamaali, 2015).

To evaluate the effects of urea concentration on enzyme activity, multiple concentrations of urea at 0.5, 1.5, 2, 3 and 4 M were prepared. Bacterial suspension of *M. lysodeikticus* (2.9 ml each) was then added to two

micro-tubes, one containing standard egg lysozyme and the other containing the sample. The optical absorption of each concentration was measured at 450 nm for 3 min at intervals of 30 s (Bazrkar and Aghamaali, 2015).

In order to study the effects of pH on enzyme activity, the following buffers were prepared: Sodium acetate (pH=4); Sodium phosphate (pH=5.8); Sodium phosphate (pH=7); Sodium phosphate (pH=6.2) and Borate sodium (pH 9). Bacterial suspension of *M. lysodeikticus* (2.9 ml) was added to two micro-tubes, one containing standard egg lysozyme and the other containing the sample. The optical density of each concentration was measured at 450 nm for 3 min at intervals of 30 s (Bazrkar and Aghamaali, 2015).

In the present study, function and activity of the enzyme at different temperatures, multiple salt concentrations and multiple pHs were studied using the enzyme kinetic method by measuring the OD (optical density) at 450 nm at intervals of 30 s for 3 min. Optical absorptions were then used in the following equation:

$$\text{Unit per ml enzyme} = \frac{(\Delta A_{450\text{nm/min Test}} - \Delta A_{450\text{ nm/min Blank}}) \text{ df}}{(0.001)(0.1)}$$

where df = dilution factor; 0.001 = change in absorbance at A<sub>450</sub> as per the Unit Definition; 0.1 = volume (in ml) of enzyme used.

Mean and percentage values were calculated and analysed by MS Excel (Microsoft, 2010).

Innate immunity is essential for protection against pathogens. Several studies reviewed the immune system of the organisms with respect to the fact that lysozyme plays an important role in innate immunity (Abdollahi *et al.*, 2016). For instance, Pabic *et al.* (2014) showed that in *Sepia officinalis*, lysozyme plays an important role in epithelial immune barriers, especially in the skin. Also, Panigrahi *et al.* (2004) showed that in case of infection, or in the presence of the pathogens, lysozyme levels increase in fish blood.

In the present study, analysis by SDS-PAGE was used for the determination of the suitable tissue for lysozyme extraction. SDS-PAGE at the reduction condition following Laemmli (1970) was used and protein bands were stained using coomassie brilliant blue (Fig. 1). The results showed that spleen is the main organ containing lysozyme enzyme in *O. mykiss*. Previous studies have shown that presence of lysozyme is higher in spleen and skin. Feng *et al.* (2011) and Caruso *et al.* (2012) demonstrated that lysozyme is essentially important in immunity against infections and increase/decrease in the concentration of lysozyme is dependent on fish species, gender, age and body weight.

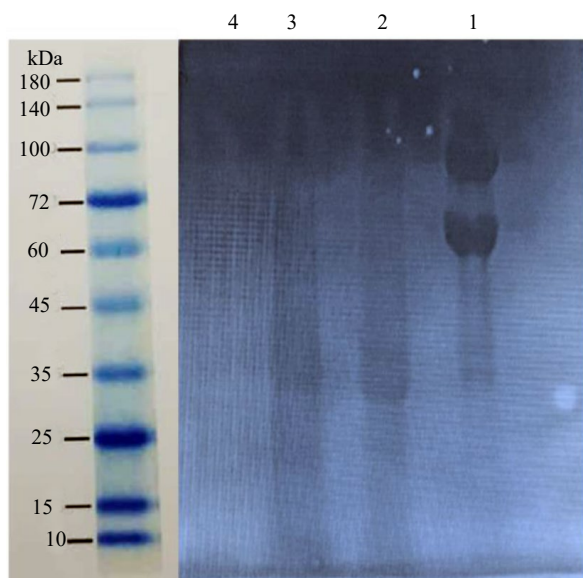


Fig. 1. SDS-PAGE image showing lysozyme content in the sampled tissues of the *O. mykiss*. Lanes 1: Liver; 2: Spleen; 3: Gill; 4: Skin mucus

Based on the results of electrophoresis, lysozyme was extracted from spleen tissue of rainbow trout and concentrated in 60% ammonium sulfate and finally, 40 mg of protein was extracted. Results showed that the optimum temperature for maximum activity of the extracted lysozyme was 60°C after that temperature, enzyme activity drastically decreased (Fig. 2). On the other hand, the optimum pH for maximum enzyme activity was found to be 5.8, beyond this pH enzyme activity decreased (Fig. 3). Results also showed that among the multiple concentrations of NaCl, the maximum activity of the enzyme was recorded at 60 mM (Fig. 4a). Furthermore, among the multiple concentrations of KCl, the maximum activity of the enzyme was recorded at 40 mM and increase in salt concentration above this, the enzyme activity decreased (Fig. 4b). Besides, the effects of multiple concentrations of MgCl<sub>2</sub> showed that the maximum activity of the extracted lysozyme was observed at 20 mM (Fig. 4c). Results also showed that the maximum activity of the extracted lysozyme was observed at 4 mM concentration of the denaturant urea (Fig. 4d).

Buonocore *et al.* (2014) studied molecular properties of the G-type lysozyme of the seabass, *Dicentrarchus labrax* and reported that the optimum activity of this lysozyme was at pH 5 and at 30°C, in presence of *M. lysodeikticus* as the substrate. In another study, Fujinami *et al.* (2014) extracted lysozyme from *Cellana nigrolineata* and showed that the activity of this lysozyme was higher than the activity of egg lysozyme by 43.3 folds. Optimum pH and temperature for the activity of

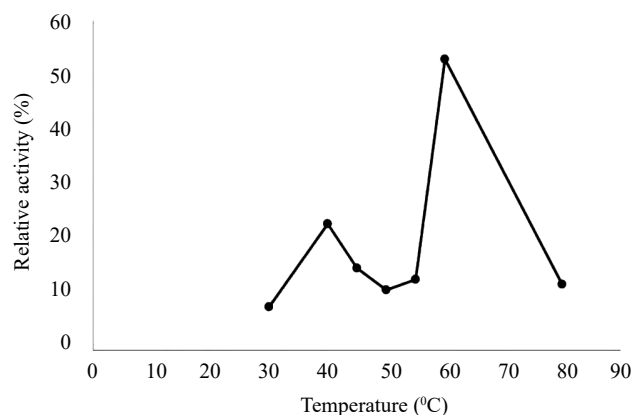


Fig. 2. Lysozyme relative activity (%) from spleen of *O. mykiss* at different temperatures

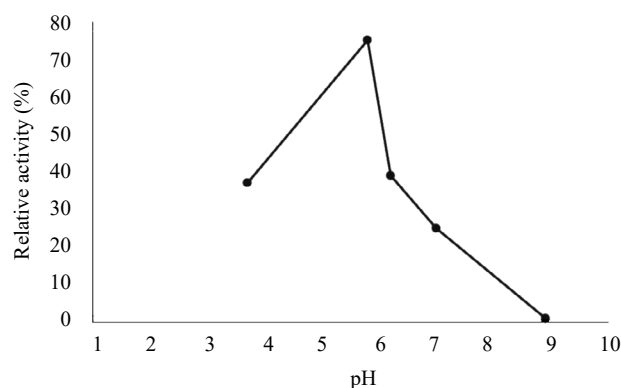


Fig. 3. Lysozyme relative activity (%) from spleen of *O. mykiss* in relation to different pH

*C. nigrolineata* lysozyme were 5 and 50°C respectively. Results of the present study showed that the optimum pH for extracted lysozyme from *O. mykiss* was 5.8. Previous studies have shown that lysozyme type C and G in Japanese flounder (Minagawa, 2001) and lysozyme type C of the *Chlamys* sp. (a marine invertebrate) (Nilsen, 1999) are active at acidic pH.

Previous studies also showed that the optimum temperature for lysozyme activity in most fish is 30-50°C (Minagawa, 2001). Some studies showed an optimum temperature below this range, such as Lysozyme G of the flounder (20-25°C) (Minagawa, 2001). In the present study, the optimum temperature for maximum activity of the lysozyme was observed at 60°C.

Activity of the *O. mykiss* lysozyme was found to be dependent on salt concentrations (NaCl, KCl and MgCl<sub>2</sub>). Results of the previous studies on lysozyme extracted from *Ruditapes philippinarum* indicated that, with increase in the concentration of NaCl from 0-70 mM and MgCl<sub>2</sub> from 0-5 mM, the enzyme activity increased (Misook, 2012).

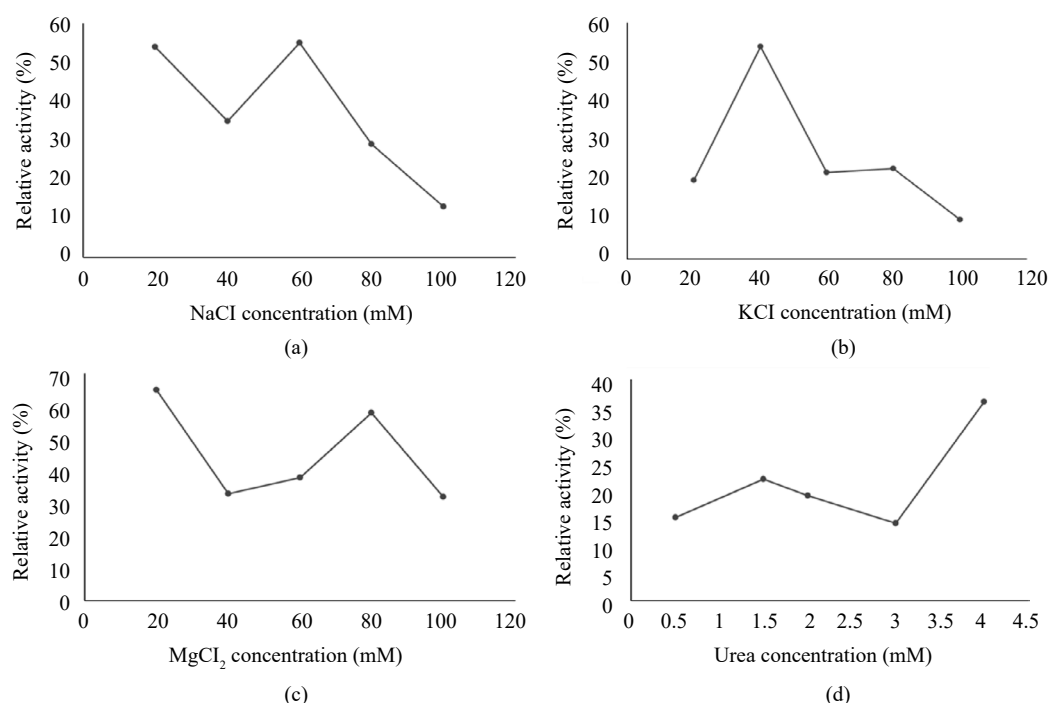


Fig. 4. Lysozyme relative activity (%) from spleen of *O. mykiss* in relation to multiple concentrations of (a) NaCl; (b) KCl; (c) MgCl<sub>2</sub> and (d) Urea

In the present study, maximum activity of the lysozyme was observed at the highest concentration of urea. Despite this finding, previous studies demonstrated that urea inhibited lysozyme activity (Sidhan, 1982; Bazrkar and Aghamaali, 2015). On the other hand, in another study on the effects of three different denaturants, guanidine thiocyanate, guanidine hydrochloride and urea, it has been observed that urea had the lowest effect on enzyme activity (Emadi and Behzadi, 2014).

Considering the results of the present study in comparison to that of previous studies, one could conclude that the lysozyme extracted from spleen of *O. mykiss* is a G-type lysozyme with its activity basically dependent on temperature, salt concentration and pH.

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