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Biochemical Characterization of Lysozyme Extracted from Common Carp, *Cyprinus carpio*

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ABSTRACT

Aim The aim of the present study was to investigate the biochemical characterizations of the lysozyme enzyme for evaluation of its importance in the immune system of the common carp, *Cyprinus carpio*.

Materials & Methods In the present study, lysozyme was extracted from the spleen of common carp, *Cyprinus carpio*. Then, partially purified by ammonium sulfate and some properties such as optimum pH and temperature as well as the effects of different salt concentrations of NaCl, $MgCl_2$, KCl, and urea on enzyme activity were evaluated. The enzyme activity was assayed using a suspension of *Micrococcus lysodeikticus* as a substrate.

Findings The optimum pH and temperature were found 4 and 50°C, respectively. Furthermore, lysozyme activity was found to be dependent on salt concentration.

Conclusion Based on the results, it's been concluded that lysozyme extracted from the spleen of the *C. carpio* has its optimum activity at high temperature and low pH condition and its activity could be continued with the presence of different salt compounds which all these are related to the environmental conditions of natural habitats of the *C. carpio* and showed that lysozyme could be one of the key factors of the immune system in this species.

Keywords Common Carp; Lysozyme; Enzymology; Immune System

CITATION LINKS

[1] Innate and adaptive immunity in teleost fish: A ... [2] Modulation of the immune system of fish by ... [3] The mucosal expression signatures of g-type lysozyme in turbot ... [4] Comparison of antimicrobial activity in the epidermal ... [5] Defense mechanisms in ... [6] Bactericidal action of lysozyme against gram-negative bacteria due to insertion of a ... [7] The distribution of mucus cells in the epidermis of the brown trout Salmo ... [8] Functions for fish ... [9] Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin ... [10] A review on the interactions between gut microbiota and innate immunity of ... [11] Immune- and enzyme histochemical characterisation of leukocyte populations within lymphoid and mucosal tissues of Atlantic halibut ... [12] Fish Immunology. The modification and manipulation of the innate immune ... [13] Innate immunity of fish ... [14] Ontogeny of the immune system of ... [15] Immunity in fish ... [16] What's new in lysozyme ... [17] Antimicrobial properties of lysozyme in relation to foodborne ... [18] Molecular cloning of an invertebrate goose-type lysozyme gene from Chlamys ... [19] cDNA cloning of the lysozyme of the white shrimp Penaeus ... [20] Antimicrobial peptides: General overview and clinical implications in human ... [21] Discovery of immune molecules and their crucial functions in ... [22] The effects of single or combined administration of galactooligosaccharide and ... [23] The non-specific immune system: Humoral ... [24] Effect of Zatana mutiflora essential oil on innate immune responses of ... [25] Effects of a severe drought on growth and ... [26] Cleavage of structural proteins during the assembly ... [27] Expression of Japanese flounder c-type lysozyme cDNA in insect ... [28] Purification and characterization of two lysozymes from rainbow trout ... [29] Amino acid sequences of lysozymes newly purified from invertebrates ... [30] Protein purification and gene isolation of chlamysin, a ... [31] Purification and characterization of lysozyme from ... [32] Characterization of the lysozyme of Mytilus ... [33] Isolation of a novel leguminous lysozyme and study on ... [34] First report of a novel plant lysozyme with both ... [35] Biochemical and antibacterial properties of lysozyme ... [36] Protein purification, cDNA cloning, and gene expression of lysozyme ... [37] Characterization and function of kuruma shrimp ... [38] Purification and characterization of lysozyme from ... [39] Trypsins from yellowfin tuna (Thunnus albacores) ... [40] Kinetic characterization of rat liver nuclear ... [41] A comparative study on the aggregating effects of guanidine ...

Introduction

Innate immunity is the first defense line against pathogens, it is an un-specific defense based on different parts of the body [1]. Un-specific compounds of the immune system are one of the important parts of the immune system and these compounds are under the effects of temperature, pH, salinity, etc. [2].

The mucus layer on the skin is the first line of defense against pathogens [3]. Fish mucus layer has several hydrolytic enzymes including lysozyme, alkalynphosphatase, Cathepsin B and proteases [4]. The fish mucus layer is secreted by goblet cells of the skin, and they made up of water and glycoproteins [5, 6]. Mucus layer of skin plays its rules in defense by preventing pathogens from attachment, ion and osmoregulation, and also acts as a reservoir of several innate immune factors such as lysozyme, immunoglobulin's, complement proteins, lectins, C-reactive protein, proteolytic enzymes, and different antibacterial proteins and peptides [7-9]. Fish mucus layer (on the skin, gill, and intestine), especially in pathogen rich environments, is a key factor in immune responses [10].

Adrenal gland and spleen are the other two parts of innate immunity in teleost fish. It has been cleared that these organs play an important role in immune responses and its vital reactions against pathogens of blood in a teleost [11]. Fish adrenal gland and spleen are composed of immune cells with multiple sizes and roles [12], such immune cells are responsible in the secretion of humoral compounds including lysozyme, Acid phosphatase (ACP) and etc. which play an important role in innate immunity [13].

Most fish lays eggs in an open environment which means embryos and larvae are facing directly with surrounding pathogens [14]. In hatching times, the immune system of the larva is not as strong and developed as in adults [15], due to this fact, it would be vital for larvae to have effective immune responses against pathogens in terms of both innate and adaptive immune responses. Most of the time larvae have been protected by immune compounds transferred from the broodstock into fertilized eggs [13].

Lysozyme has been extracted from bacterial lysis by Alexander Fleming in 1922 [16]. This enzyme is presented in all organisms with defined structural, biochemical and enzymatic characteristics [17]. Lysozyme has a hydrolytic activity against bacterial peptidoglycans and plays a vital role in the protection of an organism against pathogenic bacteria [18]. Lysozymes are amongst the oldest antibacterial peptides with a broad range of protective actions against bacteria, fungi, and viruses and they are found in vertebrates, invertebrates, plants, and even microbes [17, 19-21].

The main role of lysozymes is the elimination of pathogens through antibacterial activity [22]. Furthermore, antimicrobial activity of lysozymes is also effective in physiological pathways of digestion, anti-inflammation activities and anti-cancer pathways [21].

Lysozyme activates on gram-positive bacteria directly and on gram-negative indirectly due to their outer cell wall [17, 23]. Several parameters affected the concentration and antibacterial power of lysozyme including sexual maturity, season, water temperature, age and etc. [24].

As a global and economically important species, common carp, *Cyprinus carpio*, was selected to be investigated about lysozyme. The aim of the present study was to determine the optimum conditions for lysozyme activity in common carp as a laboratory model organism to understanding the immunity of this species.

Materials and Methods

Fish and sampling

Fish were sampled from a fish farm at Dezful, Iran, 2018. Five live adult common carp, *Cyprinus carpio*, (1-1.5Kg in body weight) were transferred to the laboratory and immediately submerged in a solution of clove powder as anesthetic procedure and then dissection and sampling of tissues have been conducted. Multiple tissues including spleen, liver, and gill, and skin mucus have been sampled and after washing with physiological serum, all samples were weight and stored in lysis buffer at -70°C [25].

Enzyme extraction using SDS-PAGE

1g of each tissue and 1g of skin mucus in lysis buffer was sonicated using BANDELIN HD 2200 and then shake for 1h using Jeio Tech SK-300. After that, samples were centrifuged at 4°C on 5000rpm using Hettich UNIVERSAL 320R. The supernatant was sampled and the sediment was thrown away.

Samples then put on SDS-PAGE gel for electrophoresis using the BioRad-Mini-PROTEAN Tetra system and considering the

results of gel electrophoresis spleen tissue was selected for continuing the study [25].

Partial purification of Enzyme (Protein sedimentation)

1g of spleen tissue was kept in 0.2M phosphate buffer with pH= 6.2. Then for completing lysis and homogenization of the cells, samples were sonicated using BANDELIN HD 2200. Samples centrifuged at 4000rpm at 4°C for 25 minutes using Hettich UNIVERSAL 320R. The supernatant with the volume of 4500ml was transferred to a new tube and kept at -70°C for the next evaluations [25].

Dialysis

1.68g of ammonium sulfate was added to 4500µl supernatant and then centrifuged at 9000rpm at 4°C for 15 minutes. The supernatant was removed and sediment used for next evaluations. 0.2M phosphate buffer sodium was added to sediment and added to a dialysis bag with 2L of 30mM ammonium bicarbonate buffer and dialyzed at 4°C for 36h.

The sample was lyophilized with Christ Alpha 1-2 LD plus and then 1mg/ml concentration of the sample was solved in 0.1M phosphate buffer at pH= 6.2 and kept at -20°C as a stock sample. Egg lysozyme of Sigma (CEWL code: L2879) was used as a standard with a concentration of 1mg/ml in 0.1M phosphate buffer [25].

Effects of temperature on enzyme activity

The suspension of bacteria, *Micrococcus lysodeikticus* ATCC 4698 from SIGMA© was modified: 0.0038g of lyophilized powder of bacteria was dissolved in 25ml of sodium phosphate buffer with the pH= 6.2.

2.9ml of bacterial suspension was transferred into a clean tube. For each temperature, two tubes were determined including standard tube containing egg lysozyme and the other one containing the sample. Each tube examined at the following temperatures and incubated in ben-Mari (Memmert WNB14) for 50 minutes: 30, 40, 45, 50, 55, 60, and 80°C.

Enzyme kinetic assayed by spectrophotometer (Spectrum SP-UV 200) at 450nm for 3 minutes with the intervals of 30s [25].

Effects of salinity concentration on enzyme activity

Three different salts of NaCl, $MgCl_2$, and KCl were used. Multiple concentrations from each salt were prepared and used as follows: 20, 40, 60, 80, and 100mM. The bacterial suspension was prepared then, using 0.0038g lyophilized *Micrococcus lysodeikticus* in 25ml sodium phosphate buffer at pH= 6.2. Modified suspension added to salt solutions and then optical absorption of each concentration was measured twice, first with standard sample (egg lysozyme) and second with observation sample at 450nm for 3 minutes with the intervals of the 30s [25].

Effects of Urea concentration on enzyme activity

Multiple concentrations of urea were prepared as follows: 0.5, 1.5, 2, 3, and 4M. The bacterial suspension was prepared then, using 0.0038g lyophilized *Micrococcus lysodeikticus* in 25ml sodium phosphate buffer at pH= 6.2. For each concentration two micro-tubes were prepared, one containing standard egg lysozyme and the other containing the observational sample. 2.9ml of bacterial suspension was added to each tube. The optical absorption of each concentration was measured at 450nm for 3 minutes with the intervals of the 30s [25].

Effects of pH on enzyme activity

In order to study the effects of pH on enzyme activity, following buffers were prepared:

- Sodium acetate (pH= 4)
- Sodium phosphate (pH= 5.8)
- Sodium phosphate (pH= 7)
- Sodium phosphate (pH= 6.2)
- Borate sodium (pH= 9)

The bacterial suspension was prepared then, using 0.0038g lyophilized *Micrococcus lysodeikticus* in 25ml sodium phosphate buffer at pH= 6.2. For each concentration two micro-tubes were prepared, one containing standard egg lysozyme and the other containing the observational sample. 2.9ml of bacterial suspension was added to each tube. The optical absorption of each concentration was measured at 450nm for 3 minutes with the intervals of the 30s [25].

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 450nm with the intervals of the 30s for 3 minutes. Optical absorptions then used in the following equation:

$$\text{Enzyme}\left(\frac{\text{Unit}}{\text{ml}}\right) = \frac{(\Delta A450nm/\min Test - \Delta A450\ nm/\min Blank)df}{(0.001)(0.1)}$$

df= dilution factor

0.001= change in absorbance at A_{450} as per the unit definition

0.1= Volume (in ml) of enzyme used

Statistical analysis

Mean, percentage values of studied samples and diagrams were calculated and depicted by Excel 2010.

Findings

Enzyme purification

After the dissection of the tissues, SDS-PAGE gel electrophoresis was used for the determination of the suitable tissue. 12% polyacrylamide gel at the reduction condition following Laemmli UK (1970) [26] was used and protein bands were appeared using coomassie brilliant blue (Figure 1).

Protein sedimentation

Spleen tissue extraction of common carp was extracted and concentrated in 60% ammonium sulfate and finally, 40mg of protein was extracted.

Optimum temperature

Results showed that enzyme activity was increased in related to an increase in temperature until the temperature of 55°C, and after that enzyme activity decreased until the temperature of 55-60°C and so on (Diagram 1).

Optimum pH

Results showed that in lower pH the activity of the enzyme was higher, and in higher pH, this activity significantly becomes lower (Diagram 2).

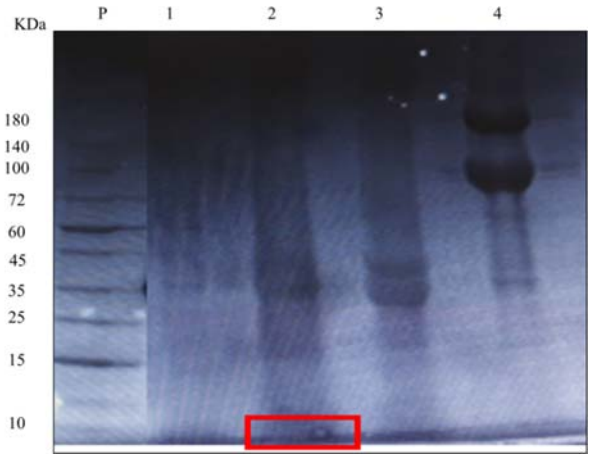

Figure 1) Electrophoresis in polyacrylamide 12% SDS-PAGE; Rectangle shows that spleen tissue had the highest lysozyme content among the sampled tissues of the common carp; (1: Liver; 2: Spleen; 3: Gill; 4: Mucus)

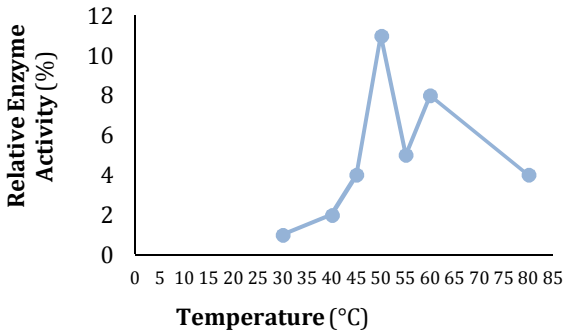

Diagram 1) The Lysozyme relative activity (%) extracted from spleen of the common carp in related to different temperatures

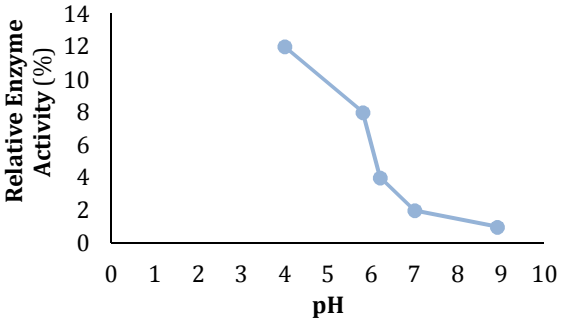

Diagram 2) The lysozyme relative activity (%) extracted from spleen of the common carp in related to different pH

Effects of salt concentration on enzyme activity

Among the multiple concentrations of NaCl, maximum activity of the enzyme was recorded at 20mM and by the increase in salt concentration, the enzyme activity becomes decrease except for the 80 and 100mM which a mild increase in enzyme activity has been observed (Diagram 3A).

Results also showed that by the increase in KCl concentration, a significant decrease in enzyme activity has been observed (Diagram 3B).

The effects of multiple concentrations of $MgCl_2$ are shown in Diagram 3. It shows that at concentrations of 20-80mM, enzyme activity increased. On the other hand, at the concentrations of 80-100mM enzyme activity decreased (Diagram 3C).

Effects of denaturant concentration on enzyme activity

By the increase in urea concentration, as a denaturant, enzyme activity decreased, except for the concentrations of 3.5-4mM which a slight increase in activity has been observed (Diagram 3D).

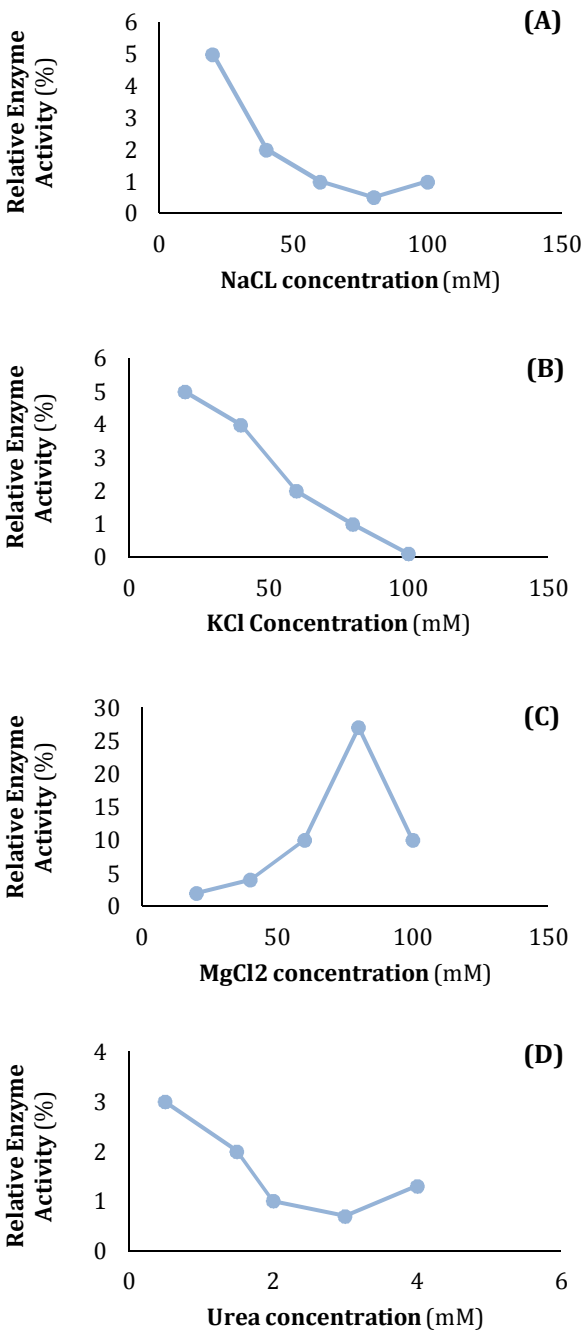

Diagram 3) The lysozyme relative activity (%) extracted from the spleen of the common carp in related to multiple concentrations of NaCl (A), KCl (B), $MgCl_2$ (C), and urea (D)

Discussion

Results of the present study showed that at 50°C, the extracted lysozyme had the highest activity. It should be considered that cyprinids are hyperthermic fish. The optimum temperature for most of the fish is about 30-50°C [27]. It revealed that the optimum temperature for the lysozyme of the rainbow trout is 45°C [28]. It has been also shown that type G lysozyme in flounder has maximum activity at 20-25°C [28].

In a previous study on *Philipino venus*, the optimum temperature for lysozyme was 75°C which is similar to the optimum temperature for Manila clam [29].

Studies also showed that the optimum temperature for lysozyme activity in Iceland scallop was 20°C [30]. This temperature for Eastern oyster was 40-45°C [31], and for Blue mussel [32], Cranberry bean [33], and Mug bean [34] has been reported as 50-55°C.

In the present study, the optimum pH for lysozyme activity has been observed as 4. This is showing that the lysozyme of the common carp is more active in acidic conditions and it is less active at neutral or basic conditions. According to previous studies, lysozyme type C and G in Japanese flounder [27], lysozyme type C of the rainbow trout [28], and lysozyme of the chlamys (a marine invertebrate) [30] are highly active at acidic pH. It's been also revealed that optimum pH for Manila clam lysozyme activity was 6-8. In the other study, the optimum pH for eastern oysters [31], scallop [35], mug bean [34], and cranberry seed [33] has been detected as 5-6. On the other hand, lysozyme extracted from Japanese flounder [27], rainbow trout [27] and eri-silkworm [36] had the maximum activity on acidic pH of 4.5-6.5.

In previous studies on Kuruma shrimp, the maximum activity of the lysozyme has been observed at pH= 6-9 [37]. Lysozyme extracted from *Philipino venus* also showed its highest activity at pH= 5.5-6.5 [38]. It's been previously revealed that changes in the pH could result in the electrical alterations in enzyme and substrate molecules, resulting in alterations in enzyme activity. It has been also showed that at strong acidic conditions enzyme activity decreases significantly [39].

In the present study, lysozyme activity of the common carp at multiple concentrations of NaCl, KCl, $MgCl_2$, and pH of 6.2 has been observed. It's been detected that lysozyme activity in common carp was increased at a concentration of 0-20mM of NaCl and by the increase of the NaCl concentration, enzyme activity was decreased. For KCl, this activity was increased from 0-60mM and then decreased at higher concentrations. About the $MgCl_2$, enzyme activity had an ascending pathway at 0-40mM and then followed a descending pathway at higher concentrations.

In a similar study on the effects of salt concentration on lysozyme activity extracted from *Ruditapes philippinarum*, it cleared that it is related to an increase in the NaCl concentration at 0-70mM and $MgCl_2$ concentration at 0-5mM, enzyme activity increased [38]. Increases in lysozyme activity at low concentrations of salt could be due to electrostatic attachment among lysozyme and bacterial cell wall and also due to interaction between polar groups of cell surface making it permeable.

In a previous study on lysozyme extracted from the kidney of Caspian Kutum, it revealed that regardless to increase in urea concentration, enzyme activity has been decreased, which this result was similar to the result of the present study [40]. Also, it's been cleared that lysozyme extracted from the liver of the rat, showed the same pattern of decreasing enzyme activity is related to increasing of the urea concentration [40]. Additionally, in the other study on the effects of three different denaturants, guanidine thiocyanate, guanidine hydrochloride, and urea, it's been cleared that urea had the lowest effect on enzyme activity [41].

Conclusion

Due to the fact that common carp belongs to the hyperthermophilic fish, it is understandable that lysozyme activity extracted from this fish increases with increases of the temperature until 50°C, and after this temperature, its activity has been decreased due to the protein structure of the enzyme. On the other hand, common carp is basically a freshwater fish, so, enzyme activity has been decreased by increases in salinity. Increases in denaturant concentration could denature the enzyme structure and end up with enzyme activity decrease. According to the results of the present study and in comparison to the findings of the previous studies, it could be concluded that the lysozyme extracted from the spleen of the *C. carpio* was a G-type lysozyme.

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