Caspian J. Environ. Sci. Vol. 16 No. 4 pp. 359~367

CJES
Caspian Journal of Environmental Sciences

[Research]

Purification and characterization of lysozyme in Persian sturgeon, *Acipenser persicus* (Borodin, 1897) from the Southwest Caspian Sea

Badan-Ara Marzdashti R.[1], Aghamaali M.R.[1]*, Varasteh A.[1], Nowruzfashkhami M.R.[2], Sabkara F.[3]

1. Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran
2. Genetic and Biotechnology Department, International Sturgeon Research Institute, Rasht, Iran
3. Department of Chemistry, Faculty of Science, Islamic Azad University of Guilan, Rasht, Iran

** Corresponding author's E-mail: aghmaali@guilan.ac.ir* (Received: April 26. 2018 Accepted: Oct. 09. 2018)

ABSTRACT

Lysozyme (N-acetylmuramide glyconohydrolase, (EC 3.2.1.17)) is a unique enzyme which cleaves the β-1,4 linkages of N-acetylmuramic and N-acetylglucosamine of the peptidoglycan, which leads to the lysis of the bacterial cell wall. Lysozyme, as a self-defense enzyme, is produced in many organs of vertebrates. The present study describes purification and characterization of lysozyme from *Acipenser persicus* (Borodin, 1897). After the extraction process, ion exchange chromatography was utilized to purify the enzyme. The SDS-PAGE analysis confirmed that the molecular weight was about 14 kDa. Moreover, some of the biochemical properties such as optimum temperature, pH and the effect of metal ions on the activity of purified enzyme were investigated. Based on the results the optimum activity and pH were obtained at 50 ˚C and 6.5 respectively. The purified lysozyme was active in the presence of different salts including NaCl (0–0.125 M), KCl (0.075–0.125 M), $MgCl_2$, and $CaCl_2$ (0.005 M). Kinetic parameters were also calculated.

Key words: Lysozyme, *Acipenser persicus*, Ion exchange chromatography, Metal ions, Optimum temperature, Catalytic efficiency.

INTRODUCTION

Lysozyme (EC 3.2.1.17) is also known as muramidase or N-acetylmuramide glycanhydrolase commonly found in nature and plays a critical role in innate immune system (Yousif *et al.* 1991). As an antibacterial agent, it hydrolysis the glycosidic bonds between the C-l of N-acetylmuramic and C-4 of N-acetylglucosamine found in the most bacterial cell wall (Salton 1957, Yousif *et al.* 1991). Besides, its comprehensive involvement in defense mechanisms such as bacteriolysis, opsonization, anti-viral and anti-neoplsatic activity in both vertebrate and invertebrates makes it a powerful antimicrobial agent commonly used as an eco-friendly and naturally occurring preservative in foods and beverages as well as a microbial cell lysis reagent (Datta 2005, Kim *et al.* 2012). Several types of lysozyme (e.g., c-, g- and i-types) have been identified in a broad range of organisms from bacteriophages to humans which show differences in their gene sequences, amino acid composition, tertiary structures, biochemical and antimicrobial properties (Kim *et al.* 2012). Lysozyme has also been studied in different marine fish species (Sankaran & Gurnani 1972, Fletcher & White 1976, Lindsay 1986). Recently, the presence of lysozyme was reported in the kidney and digestive tissues of rainbow trout *Oncorhynchus mykiss* (Lindsay 1986, Grinde *et al.* 1988) along with the kidney of Atlantic salmon *Salmo salar* (Lie *et al.* 1989,Yousif *et al.* 1991). In the present study, lysozyme was purified from the kidney

of Persian sturgeon and its molar mass was determined. Besides, the effects of temperature, pH and salts on the activity of the purified lysozyme were investigated.

MATERIALS AND METHODS

Chemicals

The chemicals with analytical grade were purchased from Merck (Darmstadt, Germany), the molecular weight marker SM 0431 and also UNOsphere$_{TM}$ S cation exchange resin were obtained from Fermentas and Bio-Rad Laboratories (Hercules, CA), respectively.

Sample preparation

The kidneys of 6 fishes were collected and transported to the Biochemistry Laboratory, University of Guilan, Rasht, Iran.

A proper amount of the sample (1.40 g) was homogenized in 5.6 mL phosphate buffer solution (PBS, pH 6.2) and the mixture was centrifuged at 9000 rpm for 20 min at 4 ˚C. Then, the supernatant was collected and stored at –20 ˚C.

Protein concentration and lysozyme activity

The protein concentration was measured using the Bradford Protein Assay and the absorbance of protein was measured at 595 nm (25 ˚C) by UV-Visible spectrophotometer (WPA Biowave II) (Xue *et al.* 2004).

The lysozyme activity was determined by the rate of *Micrococcus lysodecticus* lysis as a substrate based on the method of Shugar (Shugar 1952).

Briefly, dried cells of *M. lysodeikticus* (Sigma-Aldrich, St. Louis, MO, USA) were prepared at concentration of 0.01 w/v in PBS (pH 6.2). The aliquot (0.1 mL) of crude was added to 2.5 mL bacterial suspension.

The activity was determined from the first 2 min of linear decrease in absorbance at 450 nm and at temperature of 25 ˚C. One unit of lysozyme activity was defined as a quantity that caused a decrease in absorbance of 0.001/min of *M. lysodeikticus* suspended in 100 mM PBS (pH 6) (Shugar 1952, Kim *et al.* 2012). All measurements were performed in triplicate.

Lysozyme purification

Ammonium sulfate fractionation

A proper amount of solid ammonium sulfate was gradually added to the solution at 4 ˚C to achieve 20% saturation. The precipitated protein was isolated by centrifugation at 9000 rpm for 20 min at 4 ˚C and then re-suspended gently in 0.05 M Tris-buffer (pH 8.5). This step was repeated to obtain the solutions with 40, 60 and 80% saturation. These fractions of the precipitated proteins were dialyzed in the cold room against 0.05 M Tris-buffer (pH 8.5) over a period of 16 h to remove the residual $(NH_4)_2SO_4$. Then, the dialyzed suspension was centrifuged as described earlier to remove the insoluble particles and the presence of the target protein in the supernatant was analyzed to utilize the best fraction for the next step (Salehi *et al.* 2017).

Cation exchange chromatography

The dialyzed fraction was loaded onto a UNOsphere$_{TM}$ S column (1.5 × 20 cm) equilibrated with 0.05 M Tris-buffer (pH 8.5). The column was repeatedly eluted with 0.1, 0.2, 0.4 and 0.6 M NaCl in 0.05 M Tris-buffer (pH 8.5). The fractions were monitored for absorbance at 280 nm and assayed for lysozyme activity. The obtained samples with lysozyme activity from the peak corresponded to the 0.4 M NaCl elution were pooled and concentrated for several times using a 3 kDa cut-off cellulose acetate membrane (Millipore, Molsheim, France). The purification process was carried out at 4 ˚C. The protein concentration was measured by Bradford method and the purified samples were subjected to SDS-PAGE (Mörsky 1983; Salehi *et al.* 2017).

SDS-PAGE

The purity and approximate molecular weight of the lysozyme were estimated by SDS-PAGE under reduced condition (BioRad, Richmond, CA) with a 12.5% running gel and a 4% stacking gel by the method of Laemmli (Laemmli 1970). The protein molecular weight markers at low-range of 14.4–116 kDa were provided from Bio-Rad and used as standards to determine the molecular weight.

Enzyme characterization

The kinetic parameters of the enzyme, at different concentrations of substrate, was determined in PBS (0.1 M, pH 6.2) at 37℃ based on the method described previously (Shugar 1952, Kim *et al.* 2012).

RESULTS AND DISCUSSION

Purification and molecular weight determination

The lysozyme with lytic activity against *M. lysodeikticus* was purified from *Acipenser persicus* via ammonium sulfate fractionation and ion exchange chromatography.

At first, the enzyme was partially purified in 40% ammonium sulfate fractionation and finally was purified by ion exchange chromatography. Based on the SDS-PAGE and coomassie blue staining, the protein appeared with a single band at molecular weight of 14 kDa (Fig. 1). This molecular weight was similar to the values reported for hen egg white lysozyme (Thammasirirak *et al.* 2006). The reported molecular weights of lysozyme in literatures were between 13 to 21 kDa (Lockey & Ourth 1996; Nilsen *et al.* 1999; Schoentgen *et al.* 1982; Xue *et al.* 2004). Table 1 summarizes the purification process of the enzyme. Based on the result the final purification-fold of the lysozyme is 2.67 fold. Fig. 2 represents the chromatogram of the enzyme on cation exchange chromatography.

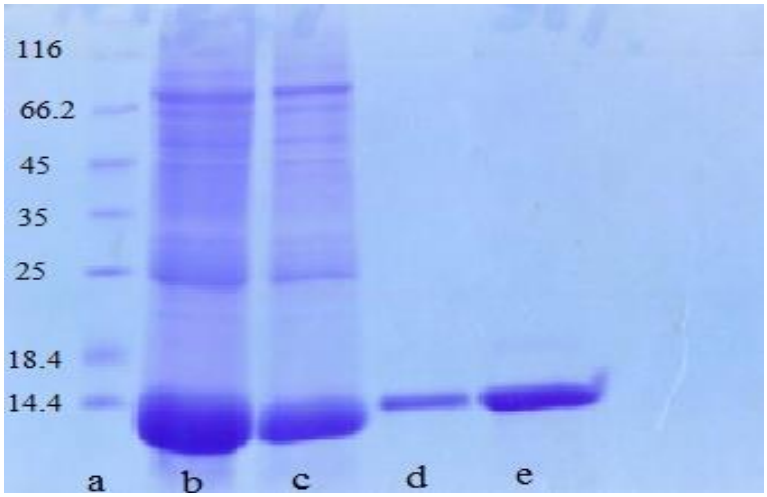

Fig. 1. SDS-PAGE of the purified sturgeon lysozyme. The molecular mass of sturgeon lysozyme was estimated about 14 kDa. a) molecular weight marker; b) crude extract; c) 40% saturation fraction; d) purified lysozyme and e) egg white lysozyme.

Table 1. Purification fold of Acipenser persicus lysozyme showed the enzyme activity in each step. The enzyme was purified 6.17 fold at least.

Purification step	Total protein (mg)	Total activity (U m L^{-1})	Protein recovery (%)	Specific activity (U m g^{-1})x10^3	Purification fold
Crude extract	2.96	133199 ± 2200	100	13.49	1
NH_4SO_4 fractionation	1.64	17504 ± 377	55.41	21.30	1.75
Ion exchange chromatography	0.60	20025 ± 430	20.25	49.97	3.70
Filtration	0.30	25026 ± 670	4.80	83.30	6.17

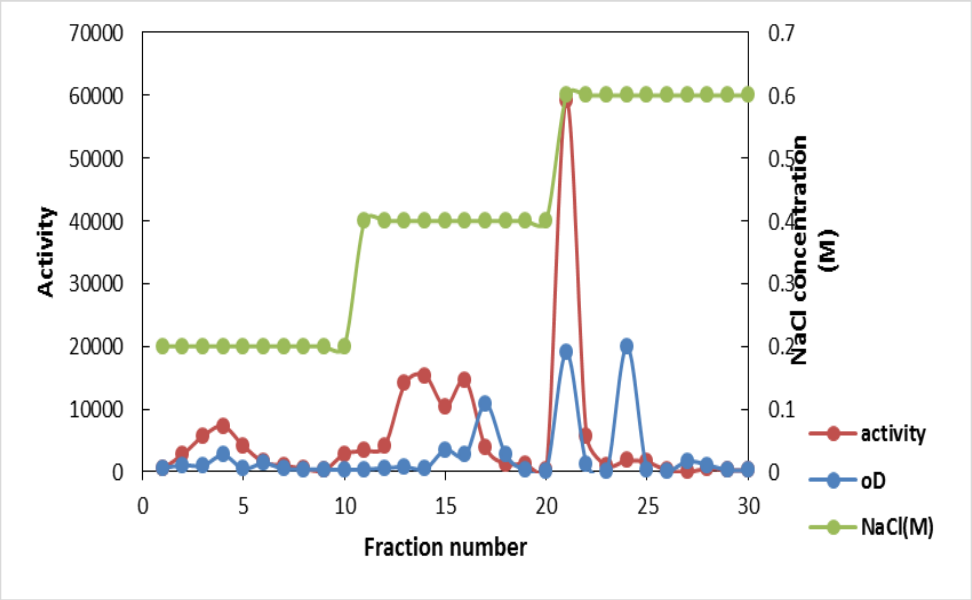

Fig. 2. The chromatogram of protein purification by ion exchange chromatography. Binding proteins were removed from the column in three steps with different NaCl concentrations (0.2, 0.4, 0.6 M), measuring the lytic activity determined that the 0.4 M fraction is the most active one.

Effect of temperature on lysozyme activity

The effect of different temperatures ranging from 25 to 60 °C on lysozyme activity is illustrated in Fig 3. As shown in this Figure the optimum temperature of the enzyme is at 50 °C. The optimum temperature of lysozyme of rainbow trout, *Oncorhynchus mykiss* was determined around 45 °C (Grinde *et al.* 1988). Based on the previous studies, the optimum temperature of most fishes was in the range of 30–50 °C (Minagawa *et al.* 2001). This indicates that lysozyme of *Acipenser persicus* was active at high temperature. As shown in Fig. 3, a decrease in the activity was observed at temperatures higher than 50 °C. It can be probably related to the thermal denaturation of the enzyme.

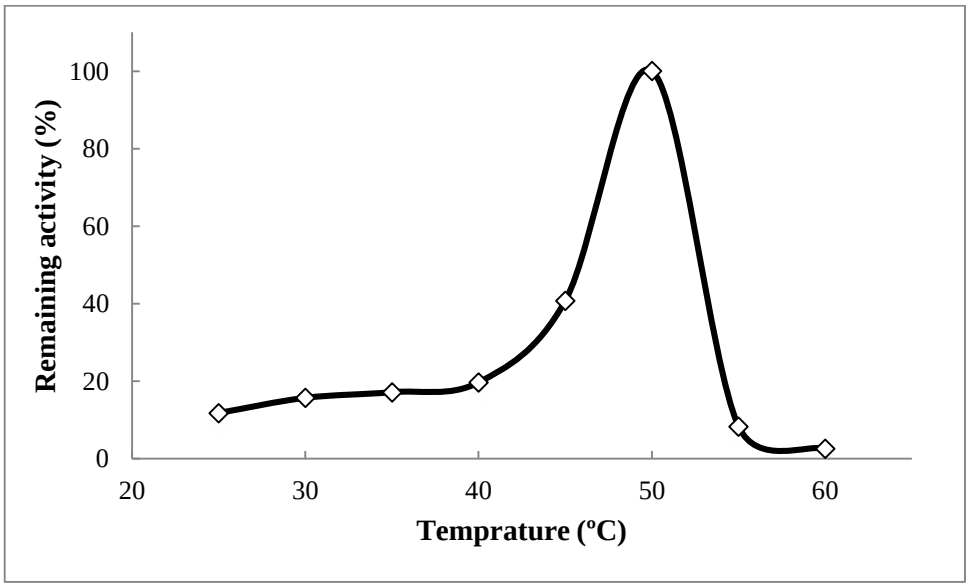

Fig. 3. Effect of temperature on lysozyme activity. The activity was measured at various temperatures (25–60 °C) in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 6.2).

Effect of pH on lysozyme activity

The optimum pH value of the purified lysozyme was 6.5, as illustrated in Fig 4. Our results are similar to those reported for the lysozyme of eastern oyster (*Crassostrea virginica*), scallop (*Patinopecten yessoensis*), mung bean (*Vigna radiate*) and cranberry seed (*Phaseolus vulgaris*) which the optimum pH value was in the range of 5.0 to 6.0 (Xue *et al.* 2004, Wang *et al*. 2005; Lee & Kim 2008; Wang *et al.* 2012). The optimal pH value for the lysozyme from Japanese flounder (*Paralichthys olivaceus*), rainbow trout (*Salmo gairdneri*) and eri-silkworm (*Samia Cynthia ricini*) was at the acidic range from 4.5 to 6.5 (Fujimoto *et al.* 2001; Grinde *et al.* 1988; Minagawa *et al.* 2001). The maximum activity of the lysozyme of the Kuruma shrimp (*Marsupenaeus japonicas*) was obtained at pH range of 6.0–9.0 (Hikima *et al.* 2003). In contrast, the activity of lysozyme from *Acipenser persicus* was low at alkaline pH values, in a way that its activity decreased substantially to 5.5% at pH 8.0, and the activity at pH 9.0 was very low. Alternation in charge distribution and conformation of substrate and enzyme can be related to changes in pH values (21). the solutions with strong acidic or alkaline pH values decrease the enzyme activity via an irreversible denaturation (Kim *et al*. 2012). It can be seen from Fig. 3, that the optimum pH value of *Acipenser persicus* lysozyme is 6.5.

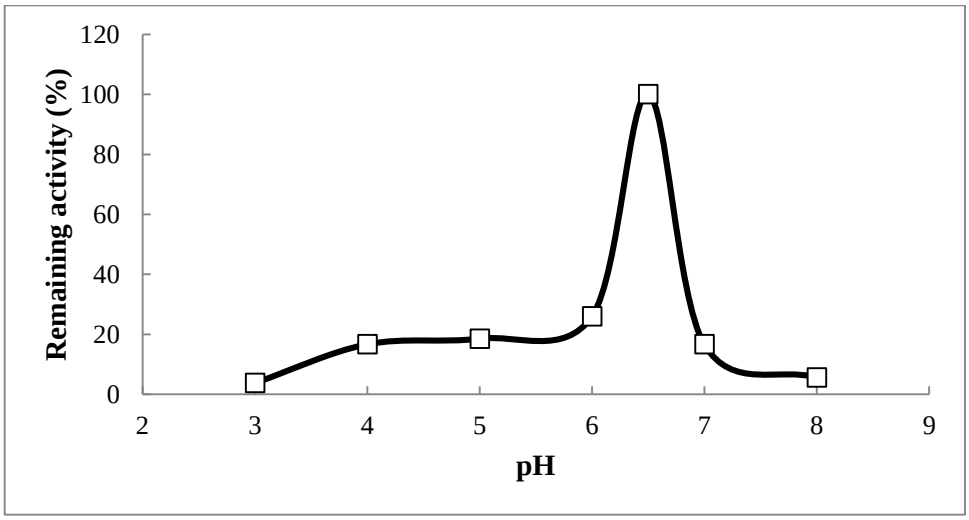

Fig. 4. Effect of pH on lysozyme activity. The activity was determined in mixed buffer solutions prepared from 0.1 M NaOH-acetic acid (pH 3.0–5.5), 0.1 M Na_2HPO_4-NaH_2PO_4 (pH 6.0–9.0) in 25 °C.

Effect of metal ions

The effects of metal ions such as Na^+, K^+, Ca^{2+} and Mg^{2+} on lysozyme activity was studied by adding NaCl, KCl, $CaCl_2$ and $MgCl_2$ at different concentrations to *M. lysodeikticus* in 0.1 M ammonium acetate buffer (pH 6.0). The activity of lysozyme was increased by increasing the concentration of NaCl from 0 to 0.125 M. The maximum activity was observed at 0.125 M and then decreased (Fig. 5A). The obtained results are in good agreement with studies on lysozyme levels in Filipino venus, *Ruditapes philippinarum* and Eastern oyster, *Crassostrea virginica*, where after using the optimum NaCl level, the enzyme activity decreased by increasing in its concentration (Datta 2005; Kim *et al.* 2012). In the case of KCl, the activity of lysozyme slightly decreased at first and then an increase was observed, while at higher concentrations, its activity decreased (Fig. 5B). In *R. philippinarum,* the lysozyme activity was stable in KCl-containing buffer at concentration range of 10–50 mM, while at higher concentrations (> 50 mM) decreased significantly (Kim *et al.* 2012). Also, the lysozyme activity in *Acipenser persicus* was slightly increased in buffer containing $MgCl_2$, up to 0.03 M, and then decreased (Fig. 5C), while in buffer containing $CaCl_2$, its activity

was increased up to 30% at 0.005 M, then decreased at higher concentrations (Fig. 5D). In Filipino venus, the activity increased slightly in buffer containing $MgCl_2$ and $CaCl_2$ in the range of 2–5 mM, whereas then decreased at 30 mM (Kim *et al.* 2012). In the case of Eastern oyster, it increased in $MgCl_2$ and $CaCl_2$ at the range of 0.005–0.035 M, while then decreased at higher concentrations (Xue *et al.* 2004).

Kinetic parameters

The kinetic parameters of K_m, k_{cat} and catalytic efficiency were also determined at the optimal temperature for the enzyme activity. Based on Lineweaver-Burk plot (Fig. 6), the data are summarized in Table 2. As showm in this Table, these parameters are achieved 0.1 (mg mL^{-1}), 41.66 (S^{-1}) and 4.166 × 10^2 (S^{-1} mg $^{-1}$ mL^{-1}), respectively.

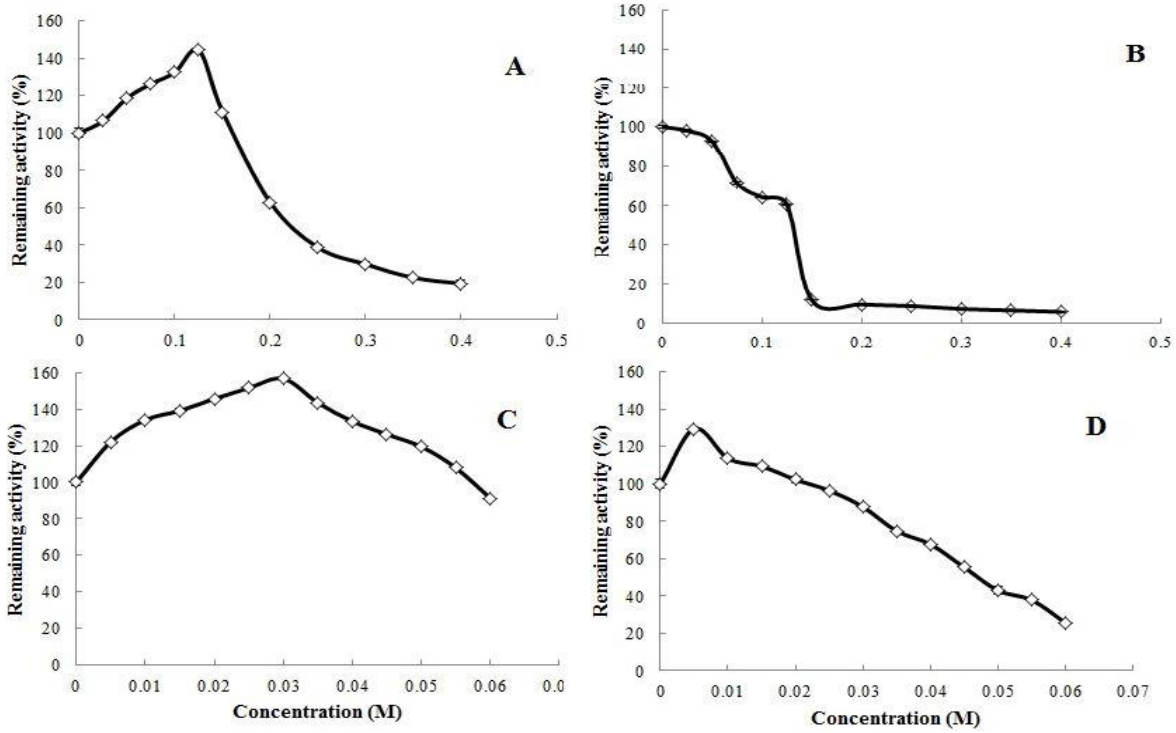

Fig. 5. Effect of monovalent metal ions (Na^+ and K^+) (A) and divalent metal ions (Mg^{2+}, Ca^{2+} and Zn^{2+}) (B) on the purified lysozyme activity in *Acipencer persicus*. Effect of metal ions on enzyme was measured by adding 0.01 mL purified lysozyme to the cocktail including NaCl, KCl, $CaCl_2$ or $MgCl_2$ at different concentrations and in *M. Lysodecticus* at 0.1 M ammonium acetate buffer.

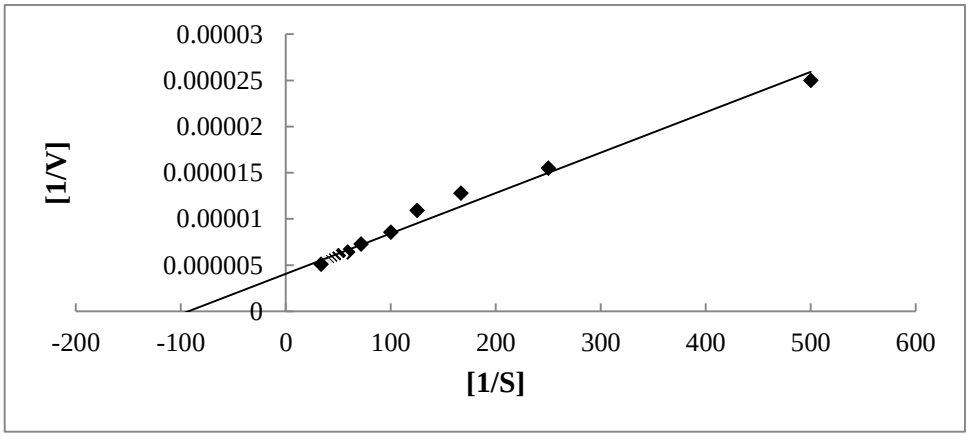

Fig. 6. Lineweaver-Burk plot for purified lysozyme. The measurements were done at different substrate concentrations (0.01v/w) in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 6.2) at optimum temperature. Base on this plot the K_m and V_{max} values were determined to be 0.1 (mg mL^{-1}) and 25000, respectively.

Table 1. Kinetic parameters of *Acipenser persicus* purified lysozyme.
These parameters measured at different concentrations of substrate at optimum temperature in 0.1 M K_2HPO4/KH_2PO4 buffer (pH 6.2).

Kinetic parameters		
K_m (mg mL^{-1})	K_{cat} (S^{-1})	(K_{cat}/K_m) $\times 10^2$
0.1	41.66	4.166

CONCLUSION

In this study, lysozyme was purified from the kidney of Persian sturgeon and then characterized. The SDS-PAGE analysis of the purified enzyme showed a single band with a molecular weight of 14 kDa. The enzyme was purified 6.17 fold. The maximum enzyme activity was obtained at 50 °C and pH 6.5. Furthermore, because of the enzyme activity at high temperature, it is possible to utilize it as a preservative in food processing for meat, fish, meat products, milk, dairy products, fruit and vegetables.

The K_m, K_{cat} and catalytic efficiency values were 0.1, 41.66 and 4.166×10^2, respectively. This enzyme could be used in pharmaceutical industries such as manufacturing process of some products like adjuvant drugs including antibiotics and analgesics for the treatment of viral and bacterial infections as well as leukemia and neoplastic diseases.

ACKNOWLEDGEMENTS

The authors would be grateful from the Research Council in the University of Guilan for their assistance in this study.

REFERENCES

Datta, S 2005, Purification of lysozyme from shell liquor of eastern oysters (*Crassostrea virginica*) and its use in antimicrobial films to preserve smoked fish.

Fletcher, TC & White, A 1976, The lysozyme of the plaice *Pleuronectes platessa* L. *Comparative Biochemistry and Physiology* Part B: *Comparative Biochemistry*, 55: 207-210.

Fujimoto, S, Toshimori-Tsuda, I, Kishimoto, K, Yamano, Y & Morishima, I 2001, Protein purification, cDNA cloning and gene expression of lysozyme from eri-silkworm, *Samia cynthia ricini*. *Comparative Biochemistry and Physiology* Part B: *Biochemistry and Molecular Biology*, 128, 709-718.

Grinde, B, Jollès, J & Jollès, P 1988, Purification and characterization of two lysozymes from rainbow trout (*Salmo gairdneri*). *The FEBS Journal,* 173, 269-273.

Hikima, S, Hikima, JI, Rojtinnakorn, J, Hirono, I & Aoki, T 2003, Characterization and function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio* species. *Gene*, 316: 187-195.

Kim, M, Park, M & Jeong, Y 2012, Purification and characterization of lysozyme from filipino venus, *Ruditapes philippinarum. Food Science and Biotechnology,* 21: 1463-1468.

Laemmli, UK 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Lee, J & Kim, S 2008, Biochemical and antibacterial properties of lysozyme purified from the viscera of scallops (*Patinopecten yessoensis*). *Journal of Food Biochemistry,* 32: 474-489.

Lie, Ø, Evensen, Ø, Sorensen, A & Froysadal, E 1989, Study on lysozyme activity in some fish species. *Diseases of Aquatic Organisms*, 6: 1-5.

Lindsay, GJ 1986, The significance of chitinolytic enzymes and lysozyme in rainbow trout (*Salmo gairdneri*) defense. *Aquaculture,* 51: 169-173.

Lockey, TD & Ourth, DD 1996, Purification and characterization of lysozyme from hemolymph of *Heliothis virescens* larvae. *Biochemical and Biophysical Research Communications,* 220: 502-508.

Minagawa, S, Hikima, JI, Hirono, I, Aoki, T & Mori, H 2001, Expression of Japanese

flounder c-type lysozyme cDNA in insect cells. *Developmental & Comparative Immunology*, 25: 439-445.

Mörsky, P 1983, Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: reexamination of reaction conditions. *Analytical biochemistry*, 128: 77-85.

Nilsen, IW, Øverbø, K, Sandsdalen, E, Sandaker, E, Sletten, K & Myrnes, B 1999, Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity. *FEBS Letters*, 464: 153-158.

Salehi, M, Aghamaali, MR, Sajedi, RH, Asghari, SM & Jorjani, E 2017, Purification and characterization of a milk-clotting aspartic protease from *Withania coagulans* fruit. *International Journal of Biological Macromolecules*, 98: 847-854.

Salton, M 1957, The properties of lysozyme and its action on microorganisms. *Bacteriological Reviews*, 21: 82.

Sankaran, K & Gurnani, S 1972, On the variation in the catalytic activity of lysozyme in fishes. *Indian Journal of Biochemistry & Biophysics*, 9: 162-165.

Schoentgen, F., Jollés, J. & Jollés, P 1982, Complete amino acid sequence of ostrich (*Struthio camelus*) egg-white lysozyme, a goose-type lysozyme. *The FEBS Journal*, 123: 489-497.

Shugar, D 1952, The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochimica et Biophysica Acta,* 8: 302-309.

Thammasirirak, S, Ponkham, P, Preecharram, S, Khanchanuan, R, Phonyothee, P, Daduang, S, Srisomsap, C, Araki, T & Svasti, J 2006, Purification, characterization and comparison of reptile lysozymes. *Comparative Biochemistry and Physiology* Part C: *Toxicology & Pharmacology*, 143: 209-217.

Wang, S, Ng, TB, Chen, T, Lin, D, Wu, J, Rao, P & Ye, X 2005, First report of a novel plant lysozyme with both antifungal and antibacterial activities. *Biochemical and Biophysical Research Communications*, 327: 820-827.

Wang, S, Ye, X & Rao, P 2012, Isolation of a novel leguminous lysozyme and study on the antifungal activity. *Food Research International,* 47: 341-347.

Xue, QG, Schey, KL, Volety, AK, Chu, FLE & LA Peyre, JF, 2004, Purification and characterization of lysozyme from plasma of the eastern oyster (*Crassostrea virginica*). *Comparative Biochemistry and Physiology* Part B: *Biochemistry and Molecular Biology*, 139: 11-25.

Yousif, A, Albright, L & Evelyn, T 1991, Occurrence of lysozyme in the eggs of coho salmon, *Oncorhynchus kisutch*. *Diseases of Aquatic Organisms*, 10, 45-49.

تخلیص و تعیین خصوصیات لایزوزیم تاسماهی ایرانی، *Asipencer persicus*

بدن آرا مرزدشتی ر.[۱]، آقامعالی م.*[۱]، وارسته ع.[۱]، نوروزفشخامی م.[۲]، سبک آرا ف.[۳]

۱- گروه زیست شناسی، دانشکده علوم پایه، دانشگاه گیلان، رشت، ایران
۲- گروه ژنتیک و بیوتکنولوژی، مرکز تحقیقات بین المللی ماهیان خاویار، رشت، ایران
۳- گروه شیمی، دانشکده علوم، دانشگاه آزاد اسلامی گیلان، رشت، ایران

(تاریخ دریافت: ۹۷/۰۲/۰۶ تاریخ پذیرش: ۹۷/۰۷/۱۷)

چکیده

لایزوزیم، N-استیل مورامید گلیکوهیدرولاز، یک آنزیم منحصر به فرد در طبیعت است که پیوند β(۱-۴) بین N-استیل مورامیک اسید و N-استیل گلوکوزآمین پپتیدوگلیکان را شکسته و موجب تجزیه شدن دیواره سلول باکتری می شود. لایزوزیم یک آنزیم خود-دفاعی است که در بسیاری از اندام‌های مهره‌داران تولید می شود. در مطالعه اخیر، به خالص‌سازی و تعیین خصوصیات لایزوزیم تاسماهی ایرانی *Acipenser persicus* پرداخته شده است. پس از استخراج، کروماتوگرافی تعویض یونی به منظور تخلیص آنزیم انجام شد. آنالیز SDS-PAGE مشخص کرد که وزن مولکولی آنزیم خالص حدود ۱۴ کیلودالتون است. علاوه بر این، برخی خصوصیات بیوشیمیایی آنزیم از جمله دما و pH بهینه فعالیت آنزیم و همچنین اثر یون های فلزی بر فعالیت آنزیم خالص بررسی شد. بر اساس نتایج، دمای بهینه فعالیت آنزیم ۵۵ درجه سانتی‌گراد و pH بهینه ۶/۵ تعیین شد. لایزوزیم خالص در حضور نمک‌های مختلف از جمله NaCl (۰,۰۰-۰/۱۲۵)، KCl (۰/۱۲۵- ۰/۰۷۵) همچنین $MgCl_2$ و $CaCl_2$ (۰/۰۰۵مولار) فعالیت خود را حفظ می کند. به علاوه، پارامترهای کینتیکی آنزیم محاسبه شد و مقادیر K_m و K_{cat} و کارایی کاتالیتیکی به‌ترتیب ۰/۱ $mg\ mL^{-1}$، ۴۱/۶۶ S^{-1} و ۴/۱۶۶×۱۰۲ به‌دست آمد.

* مؤلف مسئول