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Identification of Lysozyme Activity from Two Edible Bivalves - *Perna viridis* (Linnaeus) and *Meretrix casta* (Chemnitz)

Sumita Sharma[1*], Tanu[2] and Anil Chatterji[3]
[1]National Institute of Oceanography, Dona Paula, Goa, India
[2]Banasthali Universiti, Rajasthan
[3]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

ABSTRACT

Lysozyme activity of two commercially important edible bivalves, namely *Perna viridis* (Linnaeus) and *Meretrix casta* (Chemnitz), was analyzed against *Micrococcus luteus*. The results of this study indicated that the extract, prepared by homogenizing the mantle fluid and meat, had more lysozyme activity as compared to the acetic acid extract. The extract of *P. viridis* showed a better activity than *M. casta*. Partial purification of lysozyme was carried out using gel filtration chromatography and initial ion (IE) exchange chromatography.

Keywords: **Lysozyme activity, marine bivalves, specific activity, protein concentration**

INTRODUCTION

Lysozymes are enzymes which are widely distributed in organisms, from bacteriophages to human. The major functions of lysozymes are lysing bacterial cells by hydrolyzing the beta-1-4-linked glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of peptidoglycan, a major component of bacterial cell walls (Salton, 1957; Chipman and Sharon, 1969; Jolles, 1996). In addition, they are believed to play an important role in host defence and digestion (Allam *et al.,* 2000; Cronin *et al.*, 2001; Xue *et al.,* 2004). Therefore, they are considered as one of the important antimicrobial agents of natural origin. In contrast to the many examples of the commercial use of chicken egg white lysozyme, there is little information available on the potential use of lysozymes from other sources. It has been hypothesized that lysozymes from aquatic species may have inhibitory activity against both Gram negative and Gram positive bacteria.

Several types of lysozyme viz. *c*, *g*, *i* have been identified from a wide range of organisms (Xue *et al.,* 2004). There has been an increasing interest in the distribution and characterization of invertebrate *i*-type lysozymes (including lysozymes of bivalve molluscs) in the recent years (Bachali *et al.,* 2002; Olsen *et al.,* 2003; Takeshita *et al.*, 2003; Zavalova *et al.*, 2003; Bachali *et al.,* 2004). Lysozyme activity has been detected in the body fluids and tissues of many bivalves' molluscs. Since marine bivalve molluscs are osmoconformers and poikilotherms, they are exposed to a wide range of environmental conditions and thus lysozymes from these bivalves have been evolved to be active under different environmental conditions (Bachali *et al.,* 2004). Lysozyme of marine bivalves are active at higher salt concentrations, lower temperatures and have higher activities than the specific activities of lysozyme of egg white; this makes them better suited for food and pharmaceutical industries (Zavalova *et al.*, 2003).

The present work is an attempt to identify the lysozyme activity and partially purify the lysozyme from the whole body extracts of two edible and commercially important marine

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*Corresponding Author

bivalves, i.e. *Perna viridis* (Linnaeus) and *Meretrix casta* (Chemnitz). To the best of our knowledge, no lysozyme has been purified from these marine bivalves so far.

MATERIALS AND METHODS

Bivalves

Live specimens of *M. casta* were collected from St. Josinto Island from the intertidal zone during low tide, whereas *P. viridis,* being a sessile animal, was collected with the help of the divers from the deeper zone of the Dona Paula estuary in Goa (India) during the month of December, 2007 and March, 2008.

Extract Preparation

Animals were brought to the laboratory to be washed and de-shelled. Meat and mantle fluid was collected and divided into two equal parts: i) Acetic acid extract - One part was homogenized with chilled 2% acetic acid using a blender. The extract was centrifuged at 5000 rpm for 15 min and the supernatant containing lysozyme was collected. The second extraction was performed to further release lysozyme activity from the pellet using more than 2% acetic acid. The supernatants were collected, pooled, lyophilized and used as crude extract to check for lysozyme activity against *Micrococcus luteus.*

ii) Liquor extract - The other part was homogenized, centrifuged and supernatant was lyophilized and used as crude extract for lysozyme activity against *Micrococcus luteus.*

Determination of Protein Concentration

Protein assay (Lowry *et al.*, 1951) was carried out in triplicates to assess the concentration of protein for both the extracts of *M. casta* and *P. viridis* with bovine serum albumin as a standard protein.

Determination of Lysozyme Activity

20 µl of extract was mixed with 180 µl of bacterial suspension of *Micrococcus luteus* suspended in 0.2 M acetate buffer at pH 5.8 in a 96-well microplate at room temperature. The absorbance of the mixture was immediately measured at 450 nm with an ELISA plate reader (BioRad, Microplate Reader, Model No. 680). Absorbance was measured again 5 min after the initial reading and the decrease in the absorbance at 450 nm per min was calculated. All measurements were done in triplicates. One unit of lysozyme was defined as thet quantity which caused a decrease in the absorbance of 0.001 per min of bacterial suspension suspended in 0.2 M acetate buffer at pH 5.2 (Xue *et al.*, 2004).

Determination of Specific Activity

Specific activity was determined using the protein and lysozyme activity values:

$$\text{Specific activity} = \frac{\text{Lysozyme activity}}{\text{Protein Concentration}}$$

Partial Purification

Gel filtration chromatography - A Sephadex G-50 column was prepared and equilibrated with 0.02 M sodium acetate buffer (pH 5.0). Lyophilized extract was dissolved in sodium acetate buffer and loaded on the column. Fractions of 1 ml each were collected. The absorbance of these fractions was also monitored at 280 nm. The lysozyme activity was checked against *Micrococcus luteus.*

Ion exchange chromatography - The lyophilized active samples (McL2 and PvL2) were loaded on a SP-Sepharose FF column, equilibrated with 0.02 M sodium acetate buffer, at pH 5. The column was successively washed with 0, 0.1, 0.3 and 0.6 M of NaCl in 0.02M sodium acetate buffer pH 5.0 (Datta, 2005). The absorbance of the eluted fractions was monitored at 280 nm and lysozyme activity was checked.

SDS-PAGE – The approximate comparative molecular mass of the compounds, present in the crude lysozyme extracts, were estimated using the SDS-PAGE with a 12.0% running gel and a 4% stacking gel. The low-range (14–100 kDa) protein molecular markers, from Genei Pvt. Ltd., India, were used as the standards to calculate the molecular mass.

RESULTS AND DISCUSSION

Two different extracts, acetic acid and liquor extracts, were prepared from each animal. The total protein content was found to be more in the acetic acid extract of both the bivalves (*Fig. 1*). Surprisingly, the results of the lysozyme assay showed that the liquor extract of *P. viridis* had more lysozyme activity as compared to the acetic acid extract. On the other hand, both the

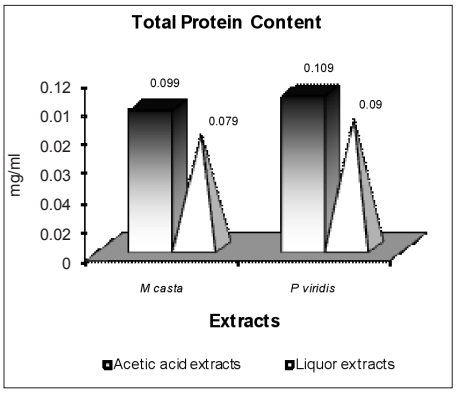

Fig. 1: Total protein content of different extracts

extracts of *M. casta* showed the same lysozyme activity. The determination of specific activities was shown to be better in the liquor extracts of both *M. casta* and *P. viridis* (Table 1). It may be possible that the acetic acid extract shows higher concentration of protein due to the fact that acetic acid also extracts proteins other than lysozymes from the animal tissues. On the other hand, liquor extract contains only those proteins which are either freely present in the mantle fluid, hemolymph or extracted due to homogenization and centrifugation (*Fig. 1*). Extracts were prepared in winter and once again at the onset of summer to see whether the different seasons have any effect on the lysozyme activity of these bivalves. It was observed that the extracts prepared in the month of March showed marked decrease in the activity and protein concentration as compared to the extracts prepared in December. Datta (2005) reported that a wide variation in the protein concentration and lysozyme activity took place in *Crassostrea virginica* due to seasonal variations. In addition, plasma lysozyme activity has been reported to be higher in winter (Chu and La Peyre, 1989).

The first step of purification using gel filtration (Sephadex G – 50) showed very encouraging results in the present study. When compared to the crude extracts, both the protein content and lysozyme activity were found to increase by ten times. The TLC carried out on these fractions confirmed the presence of proteins. The fractions were also read at 280 nm and fraction 7 of *M. casta* was observed to show the highest concentration (*Fig. 2*). On the contrary, the concentration of fraction 5 of *P. viridis* was found to be quite less (*Fig. 3*). Protein assay also showed the same pattern. Since both these fractions showed the same lysozyme activity against *M. luteus,* this might indicate the possibility that either the lysozyme present in the *P. viridis* is more potent than the one present in *M. casta* or the *M. casta* fraction has more proteins other than the lysozymes (Table 2).

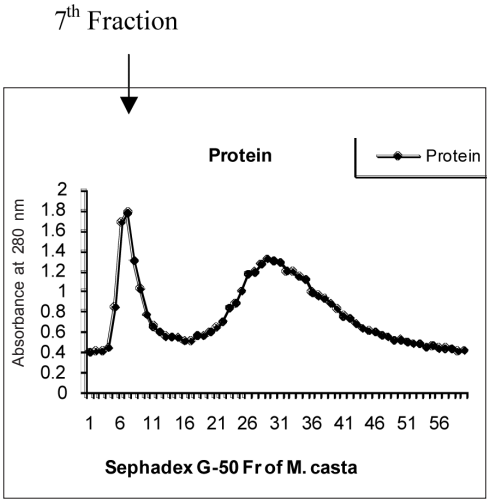

Fig. 2: Absorbance of Sephadex fractions of M. casta *at 280 nm*

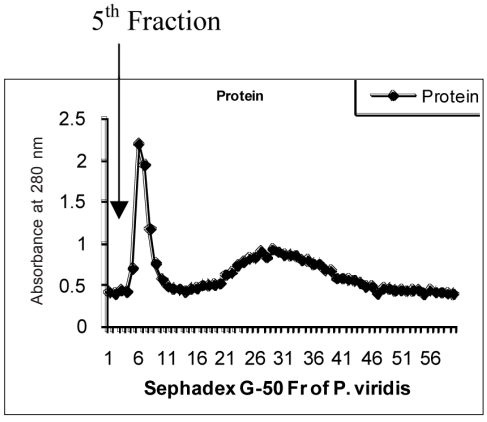

Fig. 3: Absorbance of Sephadex fractions of P. viridis *(crude and G-50 fractions)*

On the basis of the absorbance at 280 nm, the results of bioassay fractions were merged and reduced to 6 fractions in the case of *M. casta* – McL1, **McL2 (fr 7)**, McL3, McL4, McL5 and McL6, and 7 in the case of *P. viridis* - PvL1, **PvL2 (fr 5)**, PvL3, PvL4, PvL5, PvL6, PvL 7.

TABLE 1
The total protein concentration, lysozyme and specific activity of *M. casta* and *P. viridis* crude extracts

S. No	Extracts	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U/mg)
1.	Acetic acid extract of *M. casta*	12.8	0.099	1.29 x 10^2
2.	Liquor extract of *M. casta*	12.8	0.079	1.62 x10^2
3.	Acetic acid extract of *P. viridis*	14.0	0.109	1.28 x10^2
4.	Liquor extract of *P. viridis*	16.8	0.09	1.86 x10^2

TABLE 2
The total protein concentration, lysozyme and specific activity of merged G-50 fractions of *M. casta* and *P. viridis*

Merged G-50 Frs of *M. casta*	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U)	Merged G-50 Frs of *P.viridis*	Lysozyme activity (U/min/mg)	Total protein (mg/ml	Specific activity (U)
McL1 (Fr 1 - 4)	75	0.107	7.0 X10^2	PvL1 (Fr 1 - 2)	60	0.155	3.87x10^2
McL2 (Fr 7)	220	0.215	10.23X10^2	PvL2 (Fr 5)	220	0.180	12.22X10^2
McL3 (Fr 11 -12)	110	0.160	6.87X10^2	PvL3 (Fr 6 - 7)	140	0.280	5.0X10^2
McL4 (Fr 15 -19)	100	0.134	7.46X10^2	PvL4 (Fr 13 - 21)	90	0.176	5.11X10^2
McL5 (Fr 21 - 30)	150	0.180	8.33X10^2	PvL5 (25 - 28)	125	0.196	6.37X10^2
McL6 (Fr 45 - 60)	125	0.141	8.86X10^2	PvL6 (Fr 34 - 37)	80	0.198	4.04X10^2
-	-	-	-	PvL7 (Fr 47 - 55)	75	0.196	3.82X10^2

A comparative SDS PAGE of both the crude liquor extracts, McL2 and PvL2 were carried out, along with the molecular marker of 14–100 KDa. As a result, unclear bands were observed in the crude extracts of *M. casta* and *P. viridis*. However, a clear band was observed in McL2, while two were observed in PvL2, i.e. between 14 KDa and 29 KDa, indicating the possible presence of lysozymes (*Fig. 4*).

McL2 and PvL2 were further taken on a strong cation exchange column (SP Sepharose) for the next step of purification. Most of the fractions collected from McL2 did not show any activity against *M. luteus*. The fractions of PvL2 showed a better activity as compared to McL2 fractions, but these were still found to be less than the G-50 fractions (Table 3). This could be due to the fact that the solvent used (0.02 M sodium acetate buffer of pH 5 with increasing concentration of NaCl -0.00, 0.1, 0.3, and 0.6M) was not adequate to elute the lysozyme present in these samples (Xue *et al.*, 2004). In another work by Olsen *et al.* (2003), lysozyme was successfully eluted by employing a 0–0.5 M NaCl gradient in 0.05 M sodium acetate buffer (pH 5) from blue mussel (*Mytilus edulis*).

TABLE 3
Total protein concentration, lysozyme and specific activity of SP Sepharose fractions of McL2 and PvL2

SP Sepharose Fractions of McL2	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U)	SP Sepharose Fractions of PvL2	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U)
1	-	0.125		1	-	0.120	
2	-	0.123		2	-	0.121	
3	-	0.140		3	-	0.120	
4	-	0.170		4	-	0.152	
5	-	0.160		5	-	0.125	
6	-	0.165		6	-	0.135	
7	-	0.140		7	-	0.138	
8	1	0.155		8	-	0.125	
9	-	0.105		9	-	0.145	
10	-	0.140		10	-	0.145	
11	-	0.140		11	-	0.150	
12	-	0.185		12	-	0.145	
13	-	0.145		13	9	0.148	
14	-	0.145		14	-	0.140	
15	-	0.146		15	-	0.130	
16	-	0.145		16	-	0.185	
17	-	0.145		17	-	0.140	
18	-	0.130		18	-	0.140	
19	-	0.130		19	-	0.120	
20	-	0.135		20	5	0.110	
21	-	0.155		21	-	0.125	
22	-	0.160		22	-	0.128	
23	70	0.145	$4.82X10^2$	23	-	0.134	
24	80	0.145	$5.51X10^2$	24	-	0.156	
25	-	-		25	-	0.155	
26	-	0.165		26	-	0.140	
27	-	-		27	-	0.139	
28	-	0.125		28	98	0.135	$7.25X10^2$
29	-	0.120		29	-	0.140	
30	-	0.140		30	-	0.125	
31	-	0.130		31	150	0.135	$11.11X10^2$
32	-	0.185		32	155	0.135	$11.48X10^2$
33	5	0.196		33	163	0.145	$11.24X10^2$
34	100	0.180	$5.55X10^2$	34	-	0.135	
35	-	-		35	-	0.135	
36	-	-		36	-	0.120	

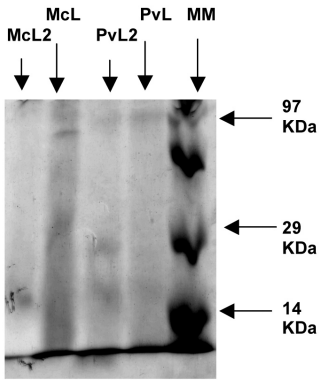

Fig. 4: Comparative SDS-PAGE of M. casta *and* P. viridis *at 280 nm*

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