The Complete Amino Acid Sequence and Enzymatic Properties of an i-Type Lysozyme Isolated from the Common Orient Clam (Meretrix lusoria)

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To determine the structure and functional relationships of invertebrate lysozymes, we isolated a new invertebrate (i)-type lysozyme from the common orient clam (Meretrix lusoria) and determined the complete amino acid sequence of two isozymes that differed by one amino acid. The determined sequence showed 65% similarity to a lysozyme from Venerupis philippinarum (Tapes japonica), and it was therefore classified as an i-type lysozyme. The lytic activities of this lysozyme were similar to those of previously reported bivalve i-type lysozymes, but unlike the V. philippinarum lysozyme, it did not exhibit an increase in activity in high ionic strength. Our data suggest that this lysozyme does not have a dimeric structure, due to the replacement of Lys108 which contributes to dimer formation in the V. philippinarum lysozyme. GlcNAc oligomer activities suggested an absence of transglycosylation activity and a higher number of subsites on this enzyme compared with hen egg lysozyme.

Key words: i-type lysozyme; amino acid sequence; invertebrate; clam

Lysozyme (EC 3.2.1.17) is widely distributed throughout the living world. This classified mainly into C (chicken), G (goose), bacterial, and phage-types. C-type lysozyme, one of the best characterized carbohydrolases, catalyzes the hydrolysis of the α-1,4-glycosidic bonds of alternating copolymers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid in bacterial cell walls, and of the homopolymer of GlcNAc, chitin.1 The three-dimensional structure of this enzyme was the first to be analyzed by X-ray crystallography2–4) and the enzyme has been studied extensively as a model protein to elucidate enzymatic function and protein stability. C-type lysozyme catalyzes not only a hydrolysis of sugar chains, but also a transglycosylation reaction that requires an acceptor oligomer instead of a water molecule at hydrolysis.5–8) The nature of the active site of hen-egg lysozyme (HEL) has been studied extensively by various methods. HEL contains six substrate binding sites, subsites A, B, C, D, E, and F. The unitary free energy changes for binding of the GlcNAc residue to the subsites have been estimated to be about −2.0, −3.0, −5.0, +4.5, −2.5, and −1.5 kcal/mol respectively.9,10) X-ray structure of the HEL-N-acetylglucosamine trimer (GlcNAc)3 complex revealed that subsite C, which provides most of the binding energy for the sugar substrate, is formed by the side chains of Trp62 and Trp63, the main chains of Asn59 and Ala107, and most importantly, a deep hydrophobic hole formed by the side chains of Ile58, Ile98, and Trp108.11) Subsite C in the HEL molecule is thought to play the most important role in substrate binding, and it plays a key role in determining the cleavage specificity of HEL. Recently, a new type of lysozyme, invertebrate type (i-type) lysozyme, has been reported.12,13) The existence of invertebrate lysozyme was first reported by Jollès and Jollès in 1975,14) and the complete structure was first determined for marine bivalve Venerupis philippinarum lysozyme (VPL), to be composed of 123 amino acids (13.8 kDa) of a size similar to that of c-type lysozyme.12,15–17) In addition, several lysozymes have been identified in shellfish, including ones from coastal bivalves belonging to the genus Mytilus,18,19) freshwater bivalve mollusk,20) sea bivalves belonging to the genera Bathytellus,21,22) and a bivalve belonging to Chlamys islandica,23,24) Furthermore, lysozymes from the starfish Asterias ruben,25) Eisenia andrei,26) Hirudo medicinalis,27,28) and Stichopus japonicas29) have also been reported. The lytic activity of bivalve lysozymes is sensitive to the salt concentrations of the assay solution.16,19) Similarly, with increasing salt concentrations, oyster and blue mussel lysozyme activities against Micrococcus luteus increased at salt concentrations of up to 0.1 M salt. At higher salt concentrations, the lytic activity decreased.21,31) This decreasing activity at higher salt conditions might have been caused by a weakness of the electrostatic interactions between the negative charges on the bacterial cell surface and the positive charges of the lysozyme, but the mechanism of salt-dependent activation remains unclear.

Multiple sequence alignment and phylogenetic analyses of six bivalve lysozymes have suggested that i-type lysozymes form a monophyletic family.32) Olsen et al.19) reported four isoforms of i-type lysozyme in blue mussel. The soft body contained one, or one major lysozyme, and three additional lysozymes were isolated from the style extract. These four lysozymes showed...
different enzymatic profile features, including their responses to pH, ionic strengths, and divalent cations. Based on these results and the profound differences among the four enzymes, Olsen et al. deduced that the multiple lysozyme activities in the blue mussel arise from proteins derived from multiple genes, instead of from an individual lysozyme variant, and that these lysozymes serve different functions in the blue mussel. Similar tissue distribution has been reported for pearl mussel, but information pertaining to the enzymatic action of i-type lysozymes is limited.

The three-dimensional structures of lysozymes from chickens,\textsuperscript{33} geese,\textsuperscript{34} and phages\textsuperscript{35} have been determined. Recently, the crystal structure of i-type lysozymes was reported for the VPL-\((\text{GlcNAc})_3\) complex to 1.6\(\text{Å}\) resolution.\textsuperscript{16} Based on structural and mutational analyses, Glu-18 and Asp-30 were found to be catalytic residues of VPL. The quaternary structure of the crystal of VPL revealed a dimer formed by electrostatic interactions between the catalytic residues in one molecule with the positive residues at the C-terminus in helix 6 of the other molecule. These findings provide evidence that the lysozyme activity of VPL is modulated by its quaternary structure, but the molecular mechanism of i-type lysozymes is not as well characterized as that of c-type lysozymes.

Here, we report the purification, primary structure analysis, and enzymatic properties of an i-type lysozyme, the common orient clam lysozyme (MLL). We compared the structure and function of MLL to those of HEL and VPL. The results revealed that these lysozymes have largely similar structures and lytic mechanisms, while MLL exhibits different salt-dependent activation compared with VPL, and different activities against GlcNAc oligomer compared with HEL.

Materials and Methods

\textbf{Chemicals.} CM-Toyopearl 650M was purchased from Tosoh (Tokyo, Japan). A C4 reverse-phase high-performance liquid chromatography (RP-HPLC) column (Hi-Pore\textsuperscript{®}Reverse Phase Column, RP304; 4.6 mm \(\times\) 250 mm) was purchased from BIO-RAD. A C18 RP-HPLC column (YMC ODS 120Å S-5; 4.6 mm \(\times\) 250 mm) was purchased from Yamamura Chemical (Kyoto, Japan).

\textbf{Materials.} Common orient clams (\textit{M. lusoria}) showing shell lengths of 40–60 mm growing in the Ariake Sea were purchased in Kumamoto, Japan and stored at \(-20^\circ\text{C}\) until use.

\textbf{Purification of lysozyme.} The samples (wet bodies, 3,155 g) were homogenized in 12.62L of 2\% acetic acid and centrifuged (8,000 rpm, 20 min). The lysozyme was re-extracted from the pellet obtained after centrifugation with 6.31L of 2\% acetic acid. The supernatant was combined and then subjected to ammonium sulfate fractionation. The lysozyme was precipitated in 20–90\% fraction of ammonium sulfate to 0.1 M, and then subjected to chromatographic purification on a CM-Toyopearl 650M cation exchange column (2 \(\times\) 28 cm) equilibrated with 50 mm phosphate buffer, pH 7.0. The column was washed with the same buffer, and the bound protein was eluted by stepwise elution with 0.3 M and 0.5 M NaCl in 50 mm phosphate buffer, pH 7.0. The lysozyme fraction was purified with a C4 RP-HPLC column by the JASCO 800 series HPLC system (Japan Spectroscopic, Hachioji, Japan). The proteins were eluted with a gradient elution method with 0.1\% trifluoroacetic acid (TFA, solvent A) and 60\% acetonitrile (MeCN) in solvent A (solvent B). A gradient of 0–60\% of solvent B was achieved in 40 min. The eluted proteins were detected by monitoring the absorbance at 280 nm.

\textbf{Lytic activity.} The lytic activity of lysozyme was assayed using the lyophilized cell wall of \textit{M. luteus} (Sigma Chemical, St. Louis, MO) as substrate. One hundred \(\mu\text{L}\) of the sample solutions was added to 3 \(\text{mL}\) of the substrate suspension in 0.1 M phosphate buffer pH 7.0, adjusted to an OD\(_{490}\) of 1.0, and then the decrease in absorption at 540 nm was measured. One enzyme unit was defined as the amount that causes a decrease of 0.1 absorbance units at 540 nm in 1 min at 25\(^\circ\text{C}\).

\textbf{Chitinase activity determination by on-gel assay.} Glycol chitin was prepared by the method of Yamada and Imoto.\textsuperscript{36} A substrate solution was prepared with 0.03\% glycol chitin in 0.05 M Tris–HCl buffer, pH 8.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli,\textsuperscript{37} with a separating gel of 12.5\% polyacrylamide containing 0.01\% glycol chitin as substrate solution. Samples were resolved without β-mercaptoethanol at 23\(^\circ\text{C}\) over 12 h. After electrophoresis, the separating gel was washed in 0.1 M Tris–HCl buffer pH 6.0 for 30 min with shaking. To measure the chitinase activity, the gel was incubated in 0.01 M Tris–HCl buffer, pH 6.0 containing 1% Triton at 37\(^\circ\text{C}\) for 60 min 2 times. After the reaction, the separating gel was stained with 0.1 M Tris–HCl buffer pH 8.9 containing 0.01% Fluorostain Brightener 28 for 5 min, and destained in water overnight. Chitinase activity bands were detected by trans-illumination at 302 nm.

\textbf{Activity for the GlcNAc oligomer.} Enzymatic activity toward (GlcNAc)\(_5\) and (GlcNAc)\(_6\) was measured by the method of Masaki et al.\textsuperscript{19} with slight modifications. Briefly, a reaction mixture containing 0.1 m\textit{M} lysozyme and 1 m\textit{M} (GlcNAc)\(_5\),\(_6\) were incubated in 10 m\textit{M} sodium acetate buffer (pH 5.0) at 50\(^\circ\text{C}\). After a given reaction time, 200\(\mu\text{L}\) of the reaction mixture was withdrawn and chilled rapidly in a KOLL KUP (Towa, Tokyo, Japan). The reaction mixture was centrifuged in Ultrafree CS3LCC (Millipore, Billerica, MA), and the filtrate was lyophilized. The dried sample was dissolved in 50\(\mu\text{L}\) of ice-cold water, and then 10\(\mu\text{L}\) of the solution was applied to a TSK Amido 80 column (4.6 mm \(\times\) 250 mm; Tosoh, Tokyo, Japan) in a JASCO 800 series HPLC system (Japan Spectroscopic, Hachioji, Japan). Elution was performed with 69\% MeCN at 30\(^\circ\text{C}\) at a flow rate of 1.0 m\textit{L}/min. Each chitooligosaccharide concentration was calculated from the peak area by monitoring the absorption at 220 nm, and estimated by interpolating the values onto standard curves obtained for authentic saccharide solutions. The relative error was defined as \((y - x)/x \times 100\), where \(x\) is the concentration of initial substrate and \(y\) is the recovered concentration of all chitooligosaccharides in (GlcNAc)\(_i\) units.

\textbf{Optimum pH and pH stability of lytic activity.} The pH optima for the lytic activity of MLL were determined and compared to that of HEL. The lytic activities against \textit{M. luteus} were measured as described above in 0.02\(\text{m}\) Britton Robinon buffer at various pH values (2.26–11.03). To assess the effect of the salt concentration on \textit{M. luteus}, the pH optima for the lytic activity of MLL were also determined in the presence of 0.15 m\textit{M} NaCl.

The pH stability of the lytic activity was analyzed and compared to that of HEL. The lytic activity was measured in 0.1 m\textit{M} phosphate buffer pH 7.0 after enzymatic solutions were pre-incubated in buffers of different pH. Enzyme solutions (6 m\textit{mol}/\text{mL}) were diluted 2-fold in 0.02\(\text{m}\) Britton Robinon buffer at various pH values and incubated at 37\(^\circ\text{C}\) for 3 h. Then, the lytic activities against \textit{M. luteus} were measured as described above.
Effect of NaCl on lytic activity. The effect of NaCl on lytic activity was measured with substrate suspensions containing 0.1, 0.2, and 0.3 M NaCl in 0.1 M phosphate buffer, pH 7.0.

Chitinase activity. Chitinase activity was measured by the method of Imoto and Yagishita,39 with some modifications. An enzyme solution (30 μL) was added to 300 μL of 0.05% glycol chitin dissolved in sodium acetate buffer, pH 5.0. After incubation for 30 min at 40 °C, 0.6 mL of 0.05% potassium ferricyanate in 0.5 M sodium carbonate was added, and the mixtures were heated in boiling water for 15 min. After the reaction, the absorbance was measured at 420 nm.

Optimum pH for chitinase activity. The pH optima for chitinase activity were determined and compared with that of HEL. Chitinase activities against glycol chitin were measured as described above in 0.02 M Britton Robinson buffer at various pH values (2.25–11.68).

Effect of NaCl on chitinase activity. The effect of NaCl on chitinase activity was measured with substrate suspensions containing 0.05, 0.10, 0.15, and 0.30 M NaCl in 0.02 M Britton Robinson buffer, pH 2.9.

Molecular weight measurement. To determine the purity of the lysozyme fractions and to obtain molecular mass information, SDS–PAGE was performed using 12.5% acrylamide gels. The protein bands were stained with Coomassie brilliant blue A250 (CBB). Bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and hen egg white lysozyme (14 kDa) were used as size standards. The molecular weights of the purified lysozymes were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a 4700a Proteomics Analyzer (Applied Biosystems, Foster City, CA).

Carboxymethylation and peptide fragmentation. Lysozymes were reduced and carboxymethylated (Cm) for structural analysis by the method of Castrofield et al.41 Briefly, 5 mg of protein was dissolved in 1.4 mL Tris–HCl pH 8.6, and then 1.2 g urea, 100 μL of ethylenediamine- netetraacetic acid (EDTA), and 33 μL of β-mercaptoethanol were added. The solution was incubated for 1 h at 37 °C under N2 gas. After the reduction, 89 mg of monooiodoacetic acid in 300 μL of 1 M NaOH was added, and the mixture was incubated for 1 h at room temperature in the dark. The reaction mixture was desalted through a Sephadex G-50 column (2 cm x 46 cm) in 0.2 M NH4OH as elution buffer. The Cm-protein fraction was pooled and then lyophilized.

Cm-protein (5 or 0.5 mg) was digested with trypsin (1/50, w/w; TR-TPCK, Cooper Biomedical, Malvern, PA) at 37 °C for 4 h in 0.1 M NH4HCO3. Chemical fragmentation was performed with cyanogen bromide (CNBr). Briefly, 0.5 mg of Cm-protein was dissolved in 350 μL of concentrated formic acid, and then 150 μL of water was added. The solution was then added 70% formic acid containing a 200-fold molar excess of CNBr over the Met residues in the lysozyme. The reaction mixture was kept at 4 °C for 24 h. Then 10 mL of water was added, and the reaction mixture was evaporated to a final volume of 1 mL. Cm-protein (0.5 mg) was also digested with arginyl endopeptidase (1/50, w/w) in 50 mM sodium phosphate buffer pH 8.0 at 37 °C for 18 h.

Separation of peptides. The digested peptides were purified with a C18 RP-HPLC column using a JASCO 800 series HPLC system. The peptides were developed with a gradient elution system with 0.1% TFA (solvent A) and 60% acetonitrile in solvent A (solvent B) (solvent system 1). A gradient of 0%–50% solvent B was achieved in 130 min. The peptides were detected at 220 nm. Some peaks were rechromatographed using a gradient system of 5 mM phosphate buffer pH 6.0 (solvent A) and 60% MeCN in solvent A (solvent B) (solvent system 2). The peptide was eluted with 100% of solvent B (solvent system 1) for protein sequence analysis with the same column. CNBr fragments were purified with the C4 RP-HPLC column, and the peptides were developed with solvent system 1.

Amino acid and sequence analyses. Peptides and Cm-protein were hydrolyzed in evacuated sealed tubes with constant boiling HCl containing 0.05% β-mercaptoethanol at 110 °C for 20 h (for peptides), or for 24 h (for Cm-protein). The hydrolysates were analyzed with an amino acid analyzer (Model L-8500A; Hitachi, Tokyo, Japan). Sequence analyses of the protein and peptides were performed using a protein sequencer (PPSQ-21A; Shimadzu, Kyoto, Japan) and Mass spectrometry, API QSTAR Pulsar i electrospray ionization Quadrupole-Time-of-flight (ESI Q/TOF) System, and 4700 Proteomics Analyzer MALDI TOF/TOF system (Applied Biosystems, Foster City, CA). For de novo sequencing by ESI Q/TOF MS/MS analysis, tryptic peptides were subjected to TOF-MS analysis, and the detected 2+ and 3+ peptide ions were then subjected to MS/MS analysis. For de novo sequencing by MALDI TOF/TOF analysis, tryptic peptides were subjected to 2-step modifications, guanidination and 4-sulfophenyl isothiocyanate (SPITC)-sulfonation, before MS analysis. Briefly, 2 μL of peptide solution was dried in a vacuum concentrator and dissolved in 1 μL of water. A 7.5 M O-methylsourea hemisulfate stock solution was prepared freshly by dissolving 50 mg of O-methylsourea hemisulfate in 50 μL of water. Anhydrous sodium hydroxide (7 M, 5.5 μL) and 1.5 μL of the O-methylsourea solution were added to the peptide solution, and the mixture was incubated at 65 °C for 15 min. The peptide after guanidination was subjected to C18 ZipTip treatment to remove the reagent. Subsequently, 10 μL of the peptide solution modified by guanidination was mixed with 10 μL of SPITC solution, which was prepared just prior to use by dissolving 1 mg of SPITC in a pyridine/ethanol solution (1:1.2 v/v/v, 100 μL). The reaction was allowed to proceed at 50 °C for 30 min. The solvent was then thoroughly evaporated in a vacuum concentrator. The resulting solid sample was dissolved in 20 μL of water. Finally, the 2-step modified peptide was subjected to MALDI TOF/TOF analysis.

Sequence analysis of the lysozyme isoform. Sequence analysis of the lysozyme isoform was performed by peptide mapping.42,43 Briefly, peptide elution was performed with a linear gradient elution system of solvent system 1. A gradient of 0 to 50% of solvent B at a flow rate of 1 mL/min for 130 min was made. Peptides were detected by monitoring the absorbance at 220 nm. Detected peptide peaks were hydrolyzed and analyzed on an amino acid analyzer. The peptides that were detected at different elution positions by map comparison and peptides of different compositions were sequenced on a protein sequencer.

Results and Discussion

Purification of the M. lusoria lysozyme

The lysozyme was purified to homogeneity by the following steps. The common orient clam (3 kg, wet bodies) was homogenized with 2% acetic acid, and the extract was subjected to ammonium sulfate fractionation (20–90% fraction). The active fraction was then subjected to 2 steps of cation exchange column chromatography, and the lysozyme fraction was obtained by elution with 0.5 M NaCl in 0.1 M phosphate buffer, pH 7.0 (Fig. 1A). This fraction was further purified by C4 RP-HPLC with two peaks of activity (Fig. 1B). These two peaks, designated M. lusoria lysozyme A and B (MLL-A and B), appeared as single bands of 14 kDa on SDS–PAGE and activity staining (Fig. 1C). The chitinase and lysozyme activity analysis showed that both MLL-A and B possess enzymatic activities. These two peaks were then lyophilized, yielding 1.1 mg and 0.3 mg of MLL-A and MLL-B, respectively (Table 1). The two forms of MLL had identical pI, molecular weight, and similar hydrophobic properties, suggesting that the isoforms likely carried microheterogeneities in view of limited numbers of amino acid substitutions.

Determination of the complete amino acid sequence of MLL-A

For structural analysis, MLL-A was reduced and carboxymethylated. It was first sequenced with the tryptic peptides, and then those tryptic peptides that overlapped with the CNBr-cleavage fragments and the
peptides obtained by arginylendopeptidase digestion were sequenced. The strategy for the determination of primary structure is summarized in Fig. 2. The tryptic peptides were separated by RP-HPLC (Fig. 2A). The 16 peaks obtained after RP-HPLC (Fig. 2A, defined by the T number) were subjected to amino acid analysis, and were found to contain peptide(s) with stoichiometric recovery. Peptides T2 and T12 were further separated by re-chromatography in the same column by solvent system 2. Amino acid sequence analysis was performed by protein sequencer and MS analysis (the peptide sequences are shown in Fig. 3). To obtain the sequences of the undetermined regions and to identify overlapping peptides, Cm-MLL was then digested with arginyl endopeptidase. The digested peptides were then purified by C18 RP-HPLC (Fig. 2B) and analyzed in the same manner as the tryptic peptides. A comparison of the three peptides (indicated by R5, R7, and R11) overlapping the sites of the tryptic peptides were identified. The sequences of the overlapping peptides were determined and the data indicated that R5, R7, and R11 overlapped tryptic peptides at positions corresponding to amino acids 112–122, 99–111, and 10–22 of MLL-A respectively (Fig. 3). To complete the overlapping, Cm-MLL-A was then cleaved by CNBr. CNBr fragments were purified by C4 RP-HPLC (Fig. 2C), and the amino acid compositions of the isolated peaks were then analyzed. One overlapping fragment (CB5) was obtained, carrying the sequence of overlapped tryptic peptides corresponding to amino acids 74–88 of MLL-A. For sequence analysis of peptides, the combined use of protein sequencer and MS has the advantage of providing sequence information from the N-terminus and the C-terminus. Two-step modification of the peptides is an optimized protocol for de novo sequencing to overcome the defects of MALDI TOF-MS. Combining these methods, we were able to identify the complete amino acid sequence of MLL-A, which is composed of 122 residues (Fig. 3). The molecular mass of 13,382 Da, calculated from the sequenced protein, coincided almost with value of 13,376 Da determined by MALDI-TOF analysis (data not shown). To determine the sequence of MLL-B, which was co-purified with MLL-A, a differential display of the HPLC peptide maps indicated that only one peptide was eluted in a

![Fig. 1. Purification of Common Orient Clam Lysozymes.](image)

Table 1. Purification of Lysozyme from *Meretrix lasoria*

<table>
<thead>
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<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (Units)</th>
<th>Specific activity (Units/mg)</th>
<th>Recovery (%)</th>
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</table>

The molecular mass of 13,382 Da, calculated from the sequenced protein, coincided almost with value of 13,376 Da determined by MALDI-TOF analysis (data not shown). To determine the sequence of MLL-B, which was co-purified with MLL-A, a differential display of the HPLC peptide maps indicated that only one peptide was eluted in a
Fig. 2. Elution Profile of Peptides and Fragment Separation.

Fig. 3. Complete Amino Acid Sequence of MLL-A.
All the sequences obtained by the sequencing analysis of MLL are summarized. Tryptic peptides (T), arginylendopeptidase digested peptides (R), and cyanogen bromide fragments (CB) are indicated. Numerals after names of peptides indicate peak number. The m/z numerals after the names of the peptides indicate that the peptide ions (m/z) were analyzed by MS. Solid lines indicate the sequenced parts of the peptides. Dotted lines indicate the regions of peptides estimated by their amino acid compositions.
The amino acid sequences determined in this study are boxed. Possible catalytic residues are indicated in red with red arrows, and the Cys residues are indicated in blue. Amino acids that contribute to substrate binding are indicated by blue arrows. The positions of the amino acids that contribute to dimer stabilization are indicated by green arrows, and the positions of those that contribute to dimer formation are indicated by yellow arrows. Proteins are indicated by their entry numbers in the Protein Knowledgebase (UniProtKB) database, as follows: Q6L6Q6, *M. luteus* (Pacific oyster); Q8ITU2, *Mytilus edulis* (Blue mussel); Q4QLOQ, *Ostrea edulis* (Eastern oyster); Q8IT75, *Penaeus monodon* (Giant tiger prawn); Q86BD1, *Macrobrachium rosenbergii* (Giant freshwater prawn); Q8I7X3, *Penaeus semisulcatus* (Green tiger prawn); and Q86SC1, *Penaeus japonicus* (Kuruma prawn).

**Enzymatic properties**

The specific activity of MLL-A against *M. luteus* was 3–4 fold higher than that of HEL, and the pH-dependence of the activity was shifted to the acidic side, with an optimum pH of 6.5 (Fig. 6A). VPL carries 4-fold higher activity than HEL, and it exhibited broad pH dependence. It appears, therefore, that MLL and VPL share similar lytic properties against the bacterial cell wall.

The chitinase activity of VPL is modulated by the formation of VPL dimers. It indicating that the dimer formed at a low salt concentration, dissociates to a monomer at high salt concentrations, increasing the availability of catalytic residues to interact with the substrate. A similar salt effect has been reported for the lytic activities of oyster and mussel lysozymes. When MLL was assayed in the presence of various concentrations of NaCl, the activity of MLL (and that of HEL) did not increase (Fig. 6A, B). These lysozymes showed decreased activity at higher pH values. The inhibition of the interaction of lysozyme and *M. luteus* by high concentration of NaCl can lower the activity in higher pH ranges. This suggests that MLL does not form a dimer at higher concentrations of NaCl.
The effects of temperature and the thermal and pH stabilities of MLL were similar to that of HEL (data not shown). The observed higher stabilities of MLL might have been due to the considerable numbers of S–S bonds that formed due to the conservation of 14 Cys resides as well as VPL. The features of the core structure of MLL are similar to those of C-type lysozymes, but it differs from the G-type lysozymes, which lack the S–S bonds.46,47)

Chitinase activity was then analyzed with glycol chitin as polymeric substrate. Activity against glycol chitin has been reported for VPL, and was indicated to have a profile similar to HEL.12) MLL, however, exhibited chitinase activity at lower pH than HEL (Fig. 6C). A possible explanation for the shift in optimum pH is interaction of the polymer substrate and the enzyme molecule, but this should be evaluated in detail by three-dimensional structural analysis. The effect of NaCl on the chitinase activity for glycol chitin of MLL showed a profile similar to that of the lytic activity, indicating that the monomeric state occurs at all the ionic strengths tested (Fig. 6B, D). This was supported by the replacement of the amino acid reported to contribute to VPL dimer formation in lower ionic strength buffers, (Lys108 to Gly in MLL). Taken together, these data suggest a loss of electrostatic interactions in MLL, resulting in the formation of a monomer.

Compared with the polymeric substrate, oligomeric substrates reflect enzymatic activity independently of


