Purification, characterization and comparison of reptile lysozymes

Sompong Thammasiriraka,⁎ Pornpimol Ponkhama, Sutthidech Preecharrama, Rathakarn Khanchanuana, Phalakorn Phonyotheea, Sakda Daduanga, Chantragan Srisomsap, Tomohiro Arakic, Jisnuson Svasti

a Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
b Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok 10210, Thailand
c Department of Bioscience, School of Agriculture, Kyushu Tokai University, Kumamoto 869-1404, Japan
d Center for Protein Structure and Function and Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Received 1 July 2005; received in revised form 3 February 2006; accepted 11 February 2006
Available online 6 March 2006

Abstract

Cation exchange column chromatography and gel filtration chromatography were used to purify four reptile lysozymes from egg white: SSTL A and SSTL B from soft shelled turtle (Trionyx sinensis), ASTL from Asiatic soft shelled turtle (Amyda cartilagenea) and GSTL from green sea turtle (Chelonia mydas). The molecular masses of the purified reptile lysozymes were estimated to be 14 kDa by SDS-PAGE. Enzyme activity of the four lysozymes could be confirmed by gel zymograms and showed charge differences on native-PAGE. SSTL A, SSTL B and ASTL had sharp pH optima of about pH 6.0, which contrasts with that of GSTL, which showed dual pH optima at about pH 6.0 and pH 8.0. The activities of the reptile lysozymes rapidly decreased within 30 min of incubation at 90 °C except for ASTL, which was more stable. Partial N-terminal amino acid sequencing and peptide mapping strongly suggested that the enzymes were C-type lysozymes. Interestingly, the mature SSTL lysozymes show an extra Gly residue at the N-terminus, which was previously found in soft-shelled turtle lysozyme. The reptile lysozymes showed lytic activity against several species of bacteria, such as Micrococcus luteus and Vibrio cholerae, but showed only weak activity to Pseudomonas aeruginosa and lacked activity towards Aeromonas hydrophila.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Amyda cartilagenea; Asiatic soft shelled turtle lysozyme; Chelonia mydas; Green sea turtle lysozyme; Lysozyme; Lysozyme type C; Purification; Reptile lysozyme; Trionyx sinensis

1. Introduction

Lysozyme (N-acetylmuramidase glycanohydrolase, (EC 3.2.1.17)) is one of the best characterized hydrolases. It cleaves β-1,4 linkages of GlcNAc homopolymer and (GlcNAc-MurNAc)n heteropolymer, which causes lysis of the bacteria containing these polymers in the cell wall. Lysozyme is considered to be a self-defense enzyme, which is produced in serum, mucus and many organs of vertebrates. Lysozymes have been classified into three distinct forms by their amino acid sequences and tertiary structures, namely, C-type (Canfield, 1963; Jollès et al., 1963; Blake et al., 1965), T4-type (Canfield, 1963; Jollès et al., 1963; Blake et al., 1965), T4-type (Inoue et al., 1970; Matthews and Remington, 1974), and G-type (Grutter et al., 1979; Simpson et al., 1980; Weaver et al., 1985). Other lysozymes have been reported including insect lysozyme found in hemolymph and midgut (Powning and Davidson, 1976; Abraham et al., 1995; Lee and Brey, 1995; Schneider, 1985; Ito et al., 1995), and fish lysozyme found in skin mucus or secretion (Grinde et al., 1988; Dautigny et al., 1991; Hikima et al., 1997; Smith et al., 2000). P and M type lysozymes have been found in different tissues of mouse, with the M type present mainly in kidney, lung, and spleen, and P type lysozyme found in the small intestine (Hammer et al., 1987; Obita et al., 2003). More recently, a novel member of lysozyme, i-type or invertebrate lysozyme is of much interest due to differences in primary structure from other reported lysozymes, with different numbers of cysteine residues and lack of conservation of the second
catalytic amino acid (the active aspartate) (Bachali et al., 2004). Invertebrate lysozyme was first reported in the starfish by Jollès and Jollès (1975). This type of enzyme has been characterized in marine bivalves, earthworm, leech, starfish and nematode (Jollès et al., 1996; Ito et al., 1999; Nilsen et al., 1999; Olsen et al., 2003; Bachali et al., 2004). The i-type lysozyme showed activity towards the bacteria in the digestive system (McHenry and Birkbeck, 1982; Haug et al., 2004).

Although lysozyme is found in many organisms, limited information is available on reptile lysozyme. Lysozymes may be important in reptiles, since they live with opportunistic bacterial infection in their environment. Reptile lysozyme was first purified from the egg white of Trionyx gangeticus Cuvier (Gayen et al., 1977). N-terminal sequencing suggested that this lysozyme was of the C-type (Jollès et al., 1977). Tortoise egg white lysozyme appears to show a different binding mode to substrate (Aschaffenburger et al., 1980; Chatterjee et al., 1983). The complete amino acid sequence and activity of soft shelled turtle lysozyme has also been described (Araki et al., 1998). In addition, lysozyme has been isolated from spiny-tailed agamid lizard (Agama caudospinosum), and appears to responsible for controlling bacterial infection (Ingram and Molyneux, 1983). Because of the limited information available on reptile lysozymes, comparative studies of lysozyme from various species of reptile will be helpful in analyzing evolution and catalytic mechanisms.

To study the diversity of lysozymes in the reptile family, we report here the separation and partial characterization of novel reptile lysozymes from the egg whites of Asiatic soft shelled turtle, soft shelled turtle, and green sea turtle which are commonly found in Thailand. The four enzymes showed varying dependence on and response to pH, and ionic strength.

2. Materials and methods

2.1. Materials

Freshly laid soft shelled turtle (Trionyx sinensis) eggs were purchased from a local breeding farm. Green sea turtle (Chelonia mydas) eggs were kindly provided by The Sea Turtle Conservation Center of Thailand. Asiatic soft shelled turtle (Amyda cartilaginea) eggs were kindly provided by The Kanchanaburi Inland Fisheries Research and Development Center, Thailand. Hen egg white lysozyme (HEWL) and Micrococcus luteus were purchased from Sigma-Aldrich (USA). Other reagents used were of the highest grade available.

2.2. Lysozyme purification

2.2.1. Purification of soft shelled turtle lysozymes

Both Asiatic soft shelled turtle lysozyme (ASTL) and soft shelled turtle egg white lysozyme (SSTL) were purified in the same manner. Egg white of each was diluted with two volumes of 0.03 M phosphate buffer, pH 7.0 and stirred at 4 °C for 30 min. The homogenate was centrifuged at 12000×g for 15 min. The supernatant was used as crude extract, and subjected to isoelectric precipitation at pHs 4.0 and 7.0. At each step of pH treatment, the solution was adjusted to the desired pH using 1 M HCl or 1 M NaOH and incubated at 4 °C for 1 h and then centrifuged at 12000×g for 30 min. The final clarified supernatant was applied to a CM-Toyopearl 650 M cation exchange column (1.3 × 90 cm), equilibrated with 0.03 M phosphate buffer, pH 7.0. The column was washed with the same buffer and the adsorbed protein was eluted with a linear gradient of NaCl concentration from 0.0 to 0.25 M in the same buffer, at a flow rate of 15 mL/h. Then the active fractions were pooled and then dialyzed against distilled water. The enzyme was lyophilized for use as the purified enzyme.

2.2.2. Purification of green sea turtle lysozyme

Green sea turtle egg white was treated with pH precipitation in the same manner as for the purification of soft shelled turtle lysozyme, until the clarified egg white was obtained as the crude enzyme solution in phosphate buffer, pH 7.0. The crude enzyme solution was applied to the CM-Toyopearl 650 M cation exchange resin, equilibrated with 0.03 M phosphate buffer, pH 7.0. The column was washed with the same buffer and eluted with a linear gradient of 0.0 to 0.3 M NaCl in the same buffer at a flow rate of 15 mL/h. The active fractions were pooled, dialyzed against distilled water and lyophilized. Then the partially purified enzyme was further subjected to gel filtration chromatography on a Sephadex G-50 column (2 × 100 cm), equilibrated with 0.05 M phosphate buffer, pH 7.0. The active fractions were then pooled and dialyzed against distilled water. The enzyme was lyophilized for use as the purified enzyme.

2.3. Enzyme assay

The lytic activity was used to assay for lysozyme activity during purification, using the lyophilized cell wall of M. luteus as a substrate. Enzyme solutions (10–100 μl) were added to 3 mL of the substrate suspension in 0.1 M phosphate buffer, pH 7.0. After adjustment to OD 1.0 at 540 nm, the reduction in absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme causing a decrease of 0.1 absorbance unit at 540 nm in 3 min of reaction at 25 °C.

2.4. Molecular weight measurement

To examine the purity of the lysozyme preparation and to obtain a molecular mass, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970), using 4% (w/v) stacking and 12.5% (w/v) resolving gel. The protein bands were stained with Coomassie brilliant blue R-250 (CBB). The low molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences, USA) was used as standard proteins.

2.5. Protein concentration

During purification, protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.
2.6. Absorption spectra

The elution profiles of protein in the column chromatography steps were followed by measuring the absorbance at 280 nm using a Shimadzu UV-160A spectrophotometer (Japan).

2.7. Refolding gel electrophoresis

*M. luteus* whole cells were labeled with the vinyl-sulfone reactive dye, Remazol Brilliant Blue (RBB) as for the synthesis of RBB-labeled starch (Rinderknecht et al., 1967) and the refolding gel was performed according to the procedure of Hardt et al. (2003). Polyacrylamide gel electrophoresis (PAGE) was performed using a 4% (w/v) stacking gel and a 12.5% (w/v) resolving gel containing 0.1% (w/v) blue *M. luteus* cells. Polyacrylamide gels and buffers contained 0.1% (w/v) SDS. Samples were boiled for 2 min in 2× sample buffer without reducing agent (62.5 mM Tris–HCl buffer, pH 6.8, 0.006% (w/v) bromophenol blue as tracking dye, 20% (v/v) glycerol, 2% (w/v) SDS). Samples of 10 μl were used for loading. Electrophoresis was performed using Mighty Small SE 250 (Hoefer Scientific Instruments, San Francisco) at a constant 120 V for 1 h until the dye front approached the lower gel margin. The gel was washed twice with distilled water for 30 min to remove SDS, incubated in a covered tray with 30 mL of refolding buffer (50 mM NaHPO4, pH 7.0 containing 1% (v/v) Triton X100), and gently shaken at 37 °C. The image of the gel was taken by digital camera (Nikon Coolpix4500, Japan) with a white background. The activity of enzyme was measured by the clear zone in an otherwise opaque bluish gel.

2.8. Native-PAGE

The procedure was performed in bed gels of 8×10×1 cm using Hoefer SE 260 system, essentially according to protocols for basic proteins (Reisfeld et al., 1962). Separation and stacking gels were made of 18% polyacrylamide at pH 4.3 and 4% polyacrylamide at pH 6.4. The electrolyte for electrode reservoirs was acetic acid-β-alanine, pH 4.1. The sample buffer contained 0.01% methylene blue in 50% (v/v) glycerol and water. The electrophoresis was run from cathode to anode for 6 h at 20 mA at 4 °C. The protein bands were stained with Coomassie Brilliant Blue R-250 (CBB).

2.9. Effect of pH on lytic activity

The pH optima for lytic activity of reptile lysozymes were examined as a function of pH and compared with HEWL. The lytic activities against *M. luteus* were measured as described above at various pHs (pH 4.0–9.0) using Miller and Golder
buffer. The reaction mixture was kept at constant ionic strength of 0.1 and 0.05 with NaCl.

2.10. Effect of temperature on lytic activity

The thermal stability was assayed in 0.1 M phosphate buffer, pH 7.0. The enzyme solution was preincubated in 90 °C for 30 min to 3 h. Then the remaining activity of enzyme was measured as described above.

2.11. Activity of reptile lysozyme against bacterial pathogen

A lysoplate assay was used to determine the lytic activity of the purified lysozymes against several bacteria: *M. luteus*, *Escherichia coli*, *S. typhi*, *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *K. pneumoniae* and *Vibrio cholerae* according to the method of Minagawa et al. (2001). In this assay, 1% agarose gel plate in 50 mM phosphate buffer, pH 6.2 containing 1 mg/mL *M. luteus* was prepared. The concentration of bacteria in each plate was adjusted to 2 absorbance units at 600 nm. Four micrograms of the purified lysozymes and standard HEWL were placed into the well. After incubating the plate at 30 °C for 24 h, the diameter of clear zone was measured (mm).

2.12. Amino acid sequence analysis

The N-terminal sequence and tryptic peptides of the purified reptile lysozymes were sequenced by Edman degradation using automated protein sequencers (PSQ-1 Shimadzu Co., Japan and ABI Model 473A Applied Biosystems Co., Japan). The lysozyme (10 mg) was reduced and carboxymethylated with iodoacetic acid according to the method of Crestfield et al. (1963) with slight modifications. After purification through a Sephadex G-50 column (1.5 × 45 cm) in 0.2 M NH$_4$OH, the Cm-lysozyme fraction was lyophilized. The Cm-lysozyme was then suspended in 0.05 M Tris–HCl buffer, pH 8.0 and digested with trypsin (1/50 w/w, TR-TPCK,

---

**Table 1**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total volume (ml)</th>
<th>Total activity (Units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (Units/mg)</th>
<th>Purification fold</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(1) SSTLs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>90</td>
<td>3139</td>
<td>283</td>
<td>11</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>87</td>
<td>2832</td>
<td>133</td>
<td>21</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>84</td>
<td>2734</td>
<td>128</td>
<td>21</td>
<td>2</td>
<td>87</td>
</tr>
<tr>
<td>CM-toyopearl of SSTL A</td>
<td>27</td>
<td>314</td>
<td>7</td>
<td>45</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>CM-toyopearl of SSTL B</td>
<td>25</td>
<td>1279</td>
<td>15</td>
<td>85</td>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td><strong>(2) ASTL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>68</td>
<td>1581</td>
<td>163</td>
<td>10</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>67</td>
<td>1454</td>
<td>102</td>
<td>14</td>
<td>1</td>
<td>92</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>67</td>
<td>1350</td>
<td>100</td>
<td>14</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>CM-toyopearl of ASTL</td>
<td>28</td>
<td>759</td>
<td>12</td>
<td>63</td>
<td>7</td>
<td>48</td>
</tr>
<tr>
<td><strong>(3) GSTL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>140</td>
<td>1432</td>
<td>371</td>
<td>4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>139</td>
<td>1508</td>
<td>228</td>
<td>7</td>
<td>2</td>
<td>105</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>137</td>
<td>1338</td>
<td>255</td>
<td>5</td>
<td>1</td>
<td>93</td>
</tr>
<tr>
<td>CM-toyopearl</td>
<td>63</td>
<td>928</td>
<td>20</td>
<td>46</td>
<td>12</td>
<td>65</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>24</td>
<td>175</td>
<td>3</td>
<td>58</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

The activity was measured by lytic activity. The protein concentration was assayed by the Lowry method.

---

Fig. 3. Effect of pH and ionic strength on the lytic activity of reptile lysozymes against *M. luteus*. *M. luteus* was dissolved in various pHs (4.0–9.0) of Miller and Golder buffer at ionic strength of 0.1 (A) and ionic strength of 0.05 (B). The reaction was measured at 25 °C for 3 min. SSTL A 10 μg/mL (dark circle), SSTL B 10 μg/mL (light square), ASTL 10 μg/mL (dark square), GSTL 10 μg/mL (dark triangle) and HEWL 10 μg/mL (light circle) were used.
Cooper Biomedical Co., Japan) at 37 °C for 4 h. The tryptic peptides were separated on a RP-HPLC column (YMC ODS 120A S-5; 4.6×250 mm, Yamamura Chemical Co., Japan) using a Shimadzu HPLC (Japan). Peptides were eluted with a linear gradient elution system with 0.1% TFA (solv. A) and 60% acetonitrile in solv. A (solv. B). A gradient 0–50% of solv. B was made in 130 min at a flow rate of 1 mL/min was made.

3. Results

3.1. Reptile lysozymes

Four reptile lysozymes were purified from the egg whites of three species *T. sinensis*, *A. cartilaginea* and *C. mydas* by a combination of ion exchange and gel filtration chromatography. For *T. sinensis*, two lysozymes designated as SSTL A and SSTL B were obtained. With Asiatic soft shelled turtle (*A. cartilaginea*), one lysozyme with high lytic activity against *M. luteus* was obtained and designated ASTL. Green sea turtle egg white showed only one homogeneous lysozyme (GSTL) after gel filtration chromatography. The SDS-PAGE patterns of purified SSTL A, SSTL B, ASTL, and GSTL were compared to HEWL as shown in Fig. 1. The final product of lysozyme purification showed a single protein band by Coomassie staining, with estimated molecular weight of ~14 kDa, for all four types. The refolding gel of the four lysozymes confirmed lysozyme activity on the gel (Fig 2A). The protein yield and enzymatic activity at each purification step are shown in Table 1. The final specific activities of the purified reptile lysozymes were 45, 85, 63
and 58 unit/mg for SSTL A, SSTL B, ASTL and GSTL, respectively.

3.2. Native-PAGE of reptile lysozymes

The four reptile lysozymes were run on native-PAGE and compared to HEWL (Fig 2B). The mobilities of SSTL A and SSTL B differed from those of ASTL and GSTL. Among the reptiles lysozymes, purified SSTL B moved most rapidly toward the cathode than other turtles’ lysozymes (Fig. 2B) and was comparable with HEWL.

3.3. Effect of pH and ionic strength on lytic activity of reptile lysozymes

Enzymatic activities of the four separated reptile lysozymes were compared to HEWL in buffer systems of varying pH (4 – 9) and ionic strength (I = 0.1 and 0.05) (Fig. 3). At ionic strength of 0.1 (Fig. 3A), SSTL A, SSTL B and ASTL showed similar narrow optimal activity at pH 6.0, unlike GSTL, which displayed dual optimal activity at pH 6.0 and pH 8.0 at both ionic strengths (Fig. 3B). In contrast, HEWL which had a broad maximum pH range from 5.5 to 7.5. SSTL A and GSTL had similar optimal pH at ionic strength of 0.05 (Fig. 3B) and 0.1 (Fig. 3A), but SSTL B and ASTL showed broad pH optima from pH 6.0 – 8.0 at ionic strength of 0.1 (Fig. 3B).

3.4. Thermal stability of reptile lysozymes

The lytic activity of SSTL A, SSTL B and GSTL decreased rapidly, with about 60% loss of activity after 30 min incubation at 90 °C, decreasing to 20 – 40% activity after 3 h incubation (Fig. 4). Interestingly, ASTL appeared to be more stable to heat, with the bulk of enzyme activity remaining after 1 h incubation at 90 °C.

3.5. Antibacterial activity

The antibacterial activities of the purified reptile lysozymes towards different bacteria were studied, and compared to those of HEWL (Fig. 5). The purified reptile lysozymes demonstrated varying degrees of growth inhibition with different bacterial strains. Reptile lysozymes showed good lytic activity against V. cholerae but weak activity against P. aeruginosa, and lacked activity against A. hydrophila, which are all reptilian pathogens. HEWL showed lytic activity against all bacteria tested except for E. coli, S. typhi and A. hydrophila (Table 2).

3.6. N-terminal sequencing and amino acid analysis of reptile lysozymes

The purified reptile lysozymes were carboxymethylated and then digested with trypsin. Peptides from the turtle lysozymes, SSTL A, SSTL B, and ASTL were separated by HPLC. All the peaks obtained with SSTL A showed almost the same elution positions as those from SSTL B. However, the peptide map of SSTLs showed minor differences to the peptide map of ASTL (data not shown). SSTL A and SSTL B showed identical N-terminal amino acid sequences up to residue 20, including a Gly residue insert at the N-terminus (Fig. 6). The amino acid sequence of SSTL A, SSTL B and ASTL and sequence alignments to other lysozymes. Alignment of reptile lysozyme sequences to Human lysozyme (HL), HEWL, STLg (soft-shelled turtle lysozyme, T. sinensis gangiticus), STLj (soft shelled turtle lysozyme, T. sinensis japonicus) and GSTL [JC7918].

Table 2

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Diameter clear zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEWL</td>
</tr>
<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>+++ (17)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>++ (12)</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>+++ (13)</td>
</tr>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>- (3)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>++ (13)</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>++ (13)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>- (3)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>++ (11)</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>- (3)</td>
</tr>
</tbody>
</table>

Lytic activity is shown as diameter of clear zone (n) in mm.

Results are summarized as: − for n=3; ± for 3<n<5; + for 5<n<10; ++ for 10<n<15; +++ for 15<n<20.
sequence of an ASTL tryptic peptide was also determined, yielding a sequence corresponding to positions 103–115 of SSTLs.

Blast search and alignment with the other C-type lysozymes indicated that the N-terminal sequence of SSTL A and SSTL B showed 100%, 75% and 70% identity with STLj, STLg and GSTL, respectively. However, the N-terminal sequence part of SSTLs showed only 50% identity to the sequences of a classical C-type lysozyme, HEWL. The ASTL peptide sequenced showed no amino acid substitutions when compared to STLj but seven, five and one amino acid substitutions when compared to HL, HEWL and GSTL, respectively.

4. Discussion

In the present study, we have purified four types of lysozymes from the egg white of three species of reptiles. To our knowledge, this is the first report of a comparative study of reptile lysozymes from various origins. The egg whites of the three species of turtles contain different numbers of lysozymes, with different characteristics. For family Trionychidae, at least two lysozyme variants were obtained, with different migration on native-PAGE, supporting the data of Gayen et al. (1977). On the other hand, in the family Cheloniidae, C. mydas showed only one lysozyme with low activity. We also screened for lysozyme activity in egg white of other reptiles, such as marine Hawksbill turtle (Eretmochelys imbricate), fresh water turtle (Cuora amboinensis) and also Eguana eugana, but no lysozyme activity was detected (not shown). Multiple isozymes have been reported in C-type lysozymes. These include duck egg white (Prager and Wilson, 1971), quail egg white (Baker and Manwell, 1967), rabbit tear (Saleh and Ibrahim, 1995), rainbow trout skin mucus (Fernandes et al., 2004b) and also the invertebrate blue mussel (Olsen et al., 2003).

The molecular weights of SSTL A, SSTL B and ASTL lysozyme were lower than that reported for egg white lysozyme from T. gangeticus Cuvier (Gayan et al., 1977). However the molecular weights were similar to the molecular mass of C-type lysozymes from hen egg white and other vertebrate lysozymes (14 kDa). The behavior of lysozymes from T. sinensis on native-PAGE is consistent with the purification profile obtained on the CM-Toyopearl column, with SSTL B being more basic and binding more strongly than SSTL A. Indeed, native-PAGE reveals that SSTL B is the most basic protein in reptilian lysozyme group. In terms of pH optima, GSTL and SSTLs have a different pH profile from the previously reported turtle lysozyme, which had a sharp pH optimum of 7.5 at 0.1 ionic strength (Gayan et al., 1977). Lysozymes from several cold blooded animals are active at acidic pH (4.5–6.5) such as Japanese flounder C-type lysozyme (Minagawa et al., 2001), rainbow trout C-type lysozyme (Grinde et al., 1988), and invertebrate lysozymes (Olsen et al., 2003; Nilsen et al., 1999).

The differences in the enzymatic properties of reptile lysozymes from HEWL may be due to differences in the number of basic amino acids, since the N-terminal sequences show only 50% identity. The effect of pH on lytic activity in lysozyme shows that basic amino acid residues may be involved in the electrostatic interaction between the positive net charge of lysozyme and the negative net charge of the cell wall (Imoto et al., 1972; Muraki et al., 1988). GSTL exhibits a dual pH optima which is the first found in a C-type lysozyme. Interestingly, dual pH optima have been reported in some plant chitinases and G type lysozymes (Thammasirirak et al., 2002) which had a core three-dimensional structure similar to C-type lysozyme (Grutter et al., 1983). This indicates that substrate specificity and the mode of action of these reptile lysozymes may depend on the details of the local active site structure, rather than their global three-dimensional fold.

Although lysozyme is well known as an antibacterial protein, with activity against Gram-positive bacteria, no reports are available on antimicrobial activity of reptile lysozymes towards bacterial pathogens. In the present work, we have studied the antimicrobial activity of four reptile lysozymes towards 3 Gram-positive bacterial and 6 Gram-negative bacteria (Table 2). Our studies indicate that with Gram-positive bacteria, all reptile lysozymes showed strong anti-microbial activity towards M. luteus, and most also had antimicrobial activity towards S. aureus and S. epidermidis, except for SSTL B which lacked activity to S. epidermidis. With Gram-negative bacteria, all reptile lysozymes, especially SSLT A and SSLT B, showed good antimicrobial activity towards V. cholerae, which is a major pathogen in the aquatic culture of shrimp. This is similar to the results with kuruma shrimp lysozyme, which showed lytic activity against several Vibrio species (Hikima et al., 2003). However, reptile lysozymes (SSTL A, ASTL and GSTL, but not SSTL B) displayed weak lytic activity toward P. aeruginosa which is associated with infectious diseases in animals. Interestingly, the most serious pathogens for reptilian species, A. hydrophila and S. typhi, were not lysed by the reptile lysozymes studied. This parallels the reports of lysozyme from aquatic animals, such as fish Japanese flounder C-type and G-type lysozymes, and the lysozyme of ayu or sweetfish, P. altivelis, which lacked antimicrobial activity towards their most serious pathogens (Minagawa et al., 2001; Hikima et al., 2001; Itami et al., 1992).

Thus, the specificities of the reptile lysozymes against bacterial substrates differ from that of HEWL. This may result from the adaptation of reptile lysozymes during the course of evolution, so that they acquire specificity towards the cell wall structures of certain bacteria found in their habitat environment. In this way, reptile egg-white lysozymes may act as antibacterial agents that provide innate immunity against certain bacteria. However, for the serious pathogens which are not lysed by lysozymes, the reptile might also have alternative immune system(s) in the egg white, such as antimicrobial peptides which have been found in rainbow trout (Smith et al., 2000; Fernandes et al., 2003, 2004a) to protect them from virulent bacteria.

The N-terminal sequences of SSTLs were homologous to STLj from T. sinensis Japonicus egg white (Araki et al., 1998). Based on their peptide maps (data not shown), SSTL A and SSTL B are likely to differ by only one or two residues and are likely to show few differences from ASTL. However, they show some differences in properties, with SSTL B having a 3-fold higher lytic activity against M. luteus than SSTL A. In addition,
SSTL A, ASTL and GSTL were more active to reptile pathogens than SSTL B. ASTL also showed similar activity to SSTLs, but showed remarkable thermostability.

In conclusion, the enzymatic properties of SSTL A, SSTL B, ASTL and GSTL showed some differences from each other and from the previously reported turtle lysozyme. Major differences in purification profile, mobility in native-PAGE and pH optimum among the reptile group implies that they differ in primary structure. To obtain more detailed information on substrate binding and catalytic action, the complete amino acid sequences and time-course of oligosaccharide hydrolysis will be further studied.

Acknowledgments

This study was supported financially by the Thailand Research Fund and KRU Research Fund. Jisnuson Svasti is a Senior Research Scholar of the Thailand Research Fund. We thank Dr. Ian Thomas for the critical review of the manuscript. Also, we thank all those who have contributed eggs, especially The Sea Turtle Conservation Center of Thailand and the Kanchanaburi Inland Fisheries Research and Development Center, Thailand.

References


Kanchanaburi Inland Fisheries Research and Development Center, Thailand.


Weaver, L.H., Grutter, M.G., Remington, S.J., Gray, T.M., Isaac, N.W., Mathews, B.W., 1985. Comparison of goose type, chicken type, and phage type lysozyme illustrates the change that occurs in both amino acid sequence and three dimensional structure during evolution. J. Mol. Evol. 21, 97–111.