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Research Article

A small chimerically bifunctional monomeric protein: *Tapes japonica* lysozyme

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Abstract. The lysozyme of the marine bilave *Tapes japonica* (13.8 kDa) is a novel protein. The protein has 46% homology with the destabilase from medicinal leech that has isopeptidase activity. Based on these data, we confirmed hydrolysis activity of *T. japonica* lysozyme against three substrates: L-γ-Glu-pNA, D-γ-Glu-pNA, and ε-(γ-Glu)-L-Lys. The optimal pH of chitinase and isopeptidase activity was 5.0 and 7.0, respectively. The isopeptidase activity was inhibited with serine protease inhibitor, but the lytic and chitinase activities were not. Moreover, only isopeptidase activity is decreased by lyophilization, but lytic and chitinase activities were not. We conclude that *T. japonica* lysozyme expresses isopeptidase and chitinase activity at different active sites.

Key words. Lysozyme; *Tapes japonica;* chitinase; isopeptidase; invertebrate lysozyme; bifunctional.

Lysozymes (EC 3.2.1.17) are widely distributed in the animal and plant kingdom [1]. Several types of lysozyme have been described: chicken (c), goose (g), bacteria, plant, phage, and invertebrate (i) types [1–6]. In particular, Jollès and Jollès first proposed the invertebrate type lysozyme in 1975 [5], and several further studies have been made on i-type lysozymes [7–10]. The major functions of lysozymes are lysing bacterial cells by hydrolyzing the beta-1-4-linked glycoside bond of the peptidoglycan on the bacterial cell wall. Thus, the biochemical function of this enzyme seems to be self-defense against bacterial infection [11, 12]. Additionally, it acts as a digestive enzyme in ruminant stomachs [13, 14].

Lytic enzymes, which belong to the lysozyme family, have been classified on the basis of organism, activity, and structure [6, 11]. The lytic function of lysozyme is to hydrolyze the beta-1-4-linked glycosidic bond between 2-acetamido-3-O-(1-carboxyethyl)-2-deoxy-D-glucopyranose (NAM) and 2-acetamido-2-deoxy-D-glucopyranose (NAG) on peptidoglycan in Gram-positive cells. Lysozyme belongs to the β-glycosidase group, and most lysozymes hydrolyze chitin (an NAG polymer). Novel types of lysozyme have been reported, but the ability of these newly identified lysozymes to hydrolyze the β-1-4-linked glycosidic bond has not yet been demonstrated. Generally, these enzymes have been classified as lysozyme-like enzymes [7, 15]. Recently, the lysozyme from the marine bivalve *Tapes japonica* has been recognized as an i-type lysozyme [8], but little is known about the structure and function of i-type lysozymes.

Lysozyme from the marine bivalve *T. japonica* is composed of 123 amino acids (13.8 kDa) [8]. The lytic activity of *T. japonica* lysozyme against *Micrococcus luteus* is 425%, the chitinase activity is 85%, and the binding ability to $(NAG)_3$ is 22% compared to hen egg lysozyme. The primary sequence of *T. japonica* lysozyme indicates 46% identity to the destabilase from medicinal leech [16].

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The destabilase from medicinal leech is an enzyme that hydrolyses ε-(γ-Glu)-Lys cross-linkage between Glu and Lys in stabilized fibrin. Destabilase has been studied in the context of thrombosis [16–18]. Similar isopeptide bonds are also found on peptidoglycan in bacterial cells. Peptidoglycans are composed of straight-chain beta-1-4-linked NAM and NAG that are annularly linked with peptide chains. These peptide chains link lactic acid residues of NAM, and there are isopeptide bonds between D-glutamic acid and lysine residues. Therefore, we investigated whether *T. japonica* lysozyme can hydrolyze an isopeptide bond, and were surprised to find that this small enzyme exhibits two enzyme activities, chitinase and isopeptidase activity, at different sites. This study may contribute to understanding enzymatic functions of i-type lysozyme.

Materials and methods

Purification of marine bivalve (*T. japonica*) lysozyme and estimation of protein concentration

Marine bivalves (*T. japonica*) were gathered on the shores near Fukuoka (Japan) and stored at −80 °C until use. *T. japonica* lysozyme was purified following a previous method described [8]. Its molecular weight was determined using SDS-PAGE and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOFMS). Protein quantity was estimated using the molecular extinction coefficients of 37,600 ($M^{-1}cm^{-1}$) for hen lysozyme and 27,000 ($M^{-1}cm^{-1}$) for *T. japonica* lysozyme.

Lysozyme activity assay

We measured the activity of lysozyme toward *M. luteus* (Sigma) cells (0.35 mg/ml) in 50 mM KH_2PO_4-NaOH buffer (pH 7). Fifty microliters of lysozyme solution (0.5 mg/ml) was added to 2 ml of *M. luteus* solution at 30 °C. The reaction was followed by the decrease in turbidity at 450 nm (Hitachi-2000S).

Chitinase assay

Chitinase activity was measured using reducing sugar produced from glycol-chitin by *T. japonica* lysozyme [19]. The enzyme and substrate concentrations were 52 µg/ml and 0.67 mg/ml, respectively.

Isopeptidase assay

The isopeptidase activity of *T. japonica* lysozyme was determined by the production of p-nitroanilide from L-γ-glutamine-p-nitroanilide (L-γ-Glu-pNA) with the absorbance at 405 nm [20]. The substrate (L-γ-Glu-pNA) was dissolved in 0.05 M 3-morpholinopropanesulfonic acid (MOPS) buffer, pH 7.0, containing 0.01 M NaCl and was prepared to a final concentration of 0.25–0.005 mg/ml (1.75 mM – 3.5 µM); the protein concentration was 0.83 mg/ml (59.8 µM). The reaction mixture of enzyme and substrate was incubated for 0 – 8 h at 37 °C. The progress of the reaction was calculated with the molar extinction coefficient of p-nitroanilide (9694 $M^{-1}cm^{-1}$) [20]. A reaction mixture without enzyme was used as a negative control. The kinetic parameters of the hydrolysis of L-γ-Glu-pNA or D-γ-Glu-pNA, catalyzed by *T. japonica* lysozyme, were calculated with an Eadie-Hofstee plot. The measurement of pH dependence for isopeptidase activity was performed in buffers of pH from 3 to 9. The protein and substrate concentrations were 0.83 mg/ml (59.8 µM) and 0.25 mg/ml (1.75 mM), respectively. We determined the isopeptidase activity for ε-(γ-Glu)-L-Lys using an amino acid analyzer. The protein and substrate concentrations were 0.83 mg/ml (59.8 µM) and 0.25 mg/ml (1.75 mM), respectively. Isopeptidase activity was inhibited with 4-(2-aminoethyl) benzensulfonyl fluoride hydrochloride (AEBSF; Nakarai). After adding 1 mM of the inhibitor, we determined isopeptidase, chitinase, and lysozyme activities.

Affinity column – chitin-coated celite

The chitin-coated celite was prepared as described previously [21]. The Chitin-coated celite column (50 × 70 mm) was equilibrated with 0.1 M Tris-HCl containing 1.0 M NaCl (pH 8.5). The purified enzyme was applied to the column and washed with equilibration buffer and eluted with 1.0 M acetic acid (0 °C).

Instrumentation

Circular dichroism (CD) spectrometry measurement was performed on a spectropolarimeter J-720W (Jasco). The protein concentration was 10 µM in 50 mM MOPS buffer (pH 7). MALDI-TOFMS measurement was performed on a PerSeptive Voyager (Jasco) using a sinapic acid matrix.

Results

Purification and analysis of *T. japonica* lysozyme

T. japonica lysozyme was isolated from the homogenate of the marine bivalve (1.223 g, without shells) and purified by cation exchange and gel filtration chromatography following the method described previously [8] (data not shown). Purified *T. japonica* lysozyme showed one band with an Mr 14 kDa on SDS-PAGE under non-reduced conditions (fig. 1 A) and an Mr of 13.8 kDa by MALDI-MS (fig. 1 B).

Isopeptidase activity of *T. japonica* lysozyme

Originally, we measured isopeptidase activity using L-γ-Glu-pNA and D-γ-Glu-pNA. These substrates are analogous substrates for isopeptide bonds in D-dimer and peptideoglycan, respectively, for bacterial cells. As shown

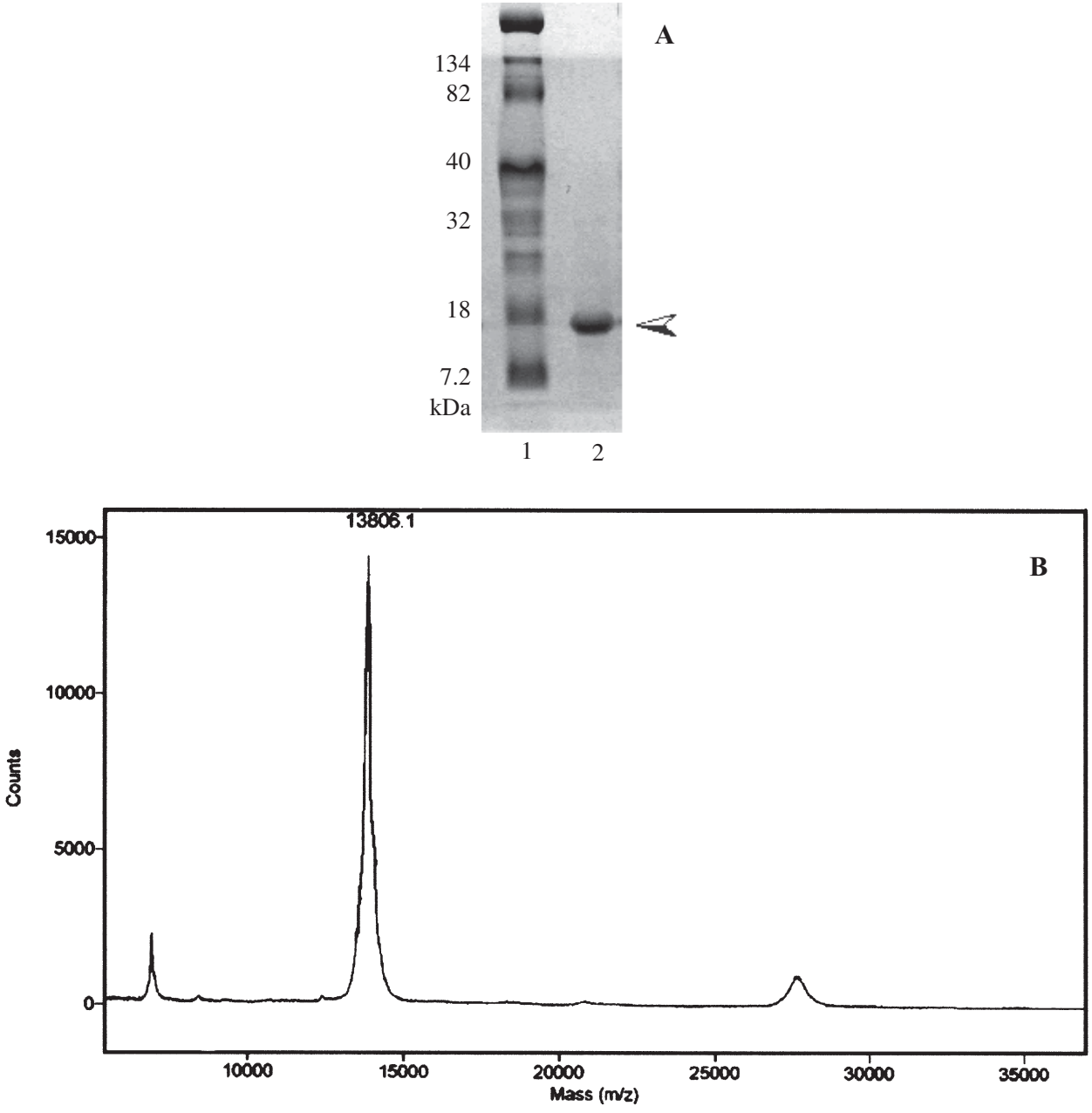

Figure 1. Identification of the molecular weight of *T. japonica* lysozyme. (*A*) SDS-PAGE (15% polyacrylamide gel) analysis of *T. japonica* lysozyme under non-reduced conditions. The molecular weights of the marker bands are 200, 116, 66, 42, 30, and 17 kDa, respectively. (*B*) MALDI-TOFMS of *T. japonica* lysozyme indicated at 13,806 Da. The theoretical value of *T. japonica* lysozyme is 13.825 kDa. There are two extra peaks at one-half (6907.82 Da) and double (27,643.3 Da) the molecular-weight peak.

in figure 2, *T. japonica* lysozyme showed distinct activities against L- or D-γ-Glu-pNA. The optimum pH was 7–8 (fig. 3). On the other hand, the optimum pH for chitinase activity was acidic. Therefore, the results of pH dependence for chitinase and isopeptidase activity showed that each enzyme activity is independent. Kinetic parameters of isopeptidase activity by *T. japonica* lysozyme were determined by the method of Eadie and Hofstee (L-γ-Glu-pNA: $K_m = 21.4$ μM, $k_{cat} = 0.2 \times 10^{-4}$ s^{-1}; D-γ-Glu-pNA: $K_m = 83.6$ μM, $k_{cat} = 0.54 \times 10^{-4}$ s^{-1}). *T. japonica* lysozyme showed a distinct activity for ε-(γ-Glu)-L-Lys, an analogous specific substrate (fig. 4).

Inhibition assay

Since the isopeptidase activity by *T. japonica* lysozyme was inhibited by AEBSF, it was shown to be a serine protease type (table 1). However, *T. japonica* lysozyme retained lytic and chitinase activity after the

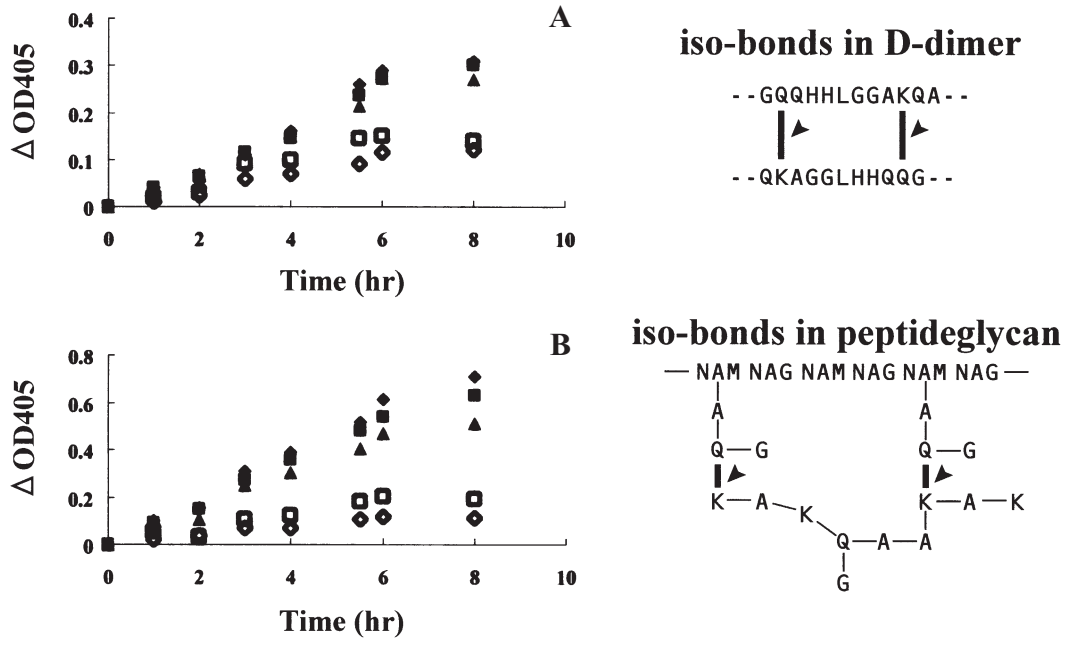

Figure 2. Assay of isopeptidase activity for *T. japonica* lysozyme against L- (*A*) or D-γ-Glu-pNA (*B*). *T. japonica* lysozyme concentration was 0.83 mg/ml (59.8 μM). Substrate (L-γ-Glu-pNA and D-γ-Glu-pNA) concentrations were 0.25–0.005 mg/ml (1.75 mM–3.5 μM), ◆, 0.25 mg/ml; ■, 0.1 mg/ml; ▲, 0.05 mg/ml; ◇, 0.01 mg/ml, □, 0.005 mg/ml. Buffer only was employed for the negative control reaction. Reactions were conducted in 0.05 M MOPS buffer (pH 7.0) at 37 °C for 8 h. Isopeptide bonds in D-dimer and peptidoglycan are indicated by arrow heads. (*A*)

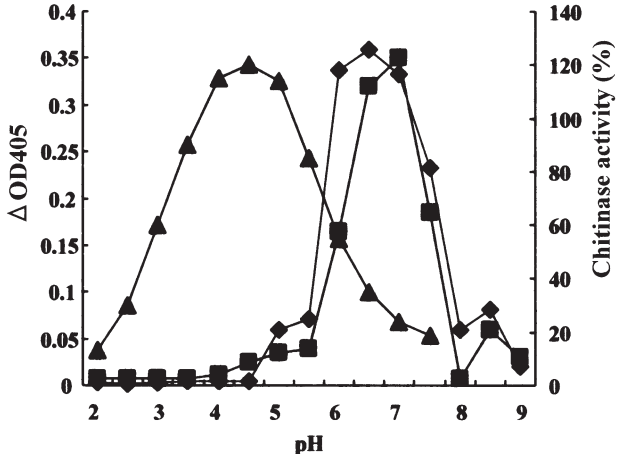

Figure 3. The pH dependence of *T. japonica* lysozyme activity against L- and D-γ-Glu-pNA. The activity toward L- (◆) or D-γ-Glu-pNA (■) was examined at pH 3.0–9.0 at 37 °C. The activity is expressed by a rise of OD 405 nm at 4 or 2 h for the L or D substrate, respectively. The enzyme and substrate concentrations were 0.83 mg/ml (59.8 μM) and 0.25 mg/ml (1.75 mM), respectively. The optimal pH of isopeptidase activity was 7.0–8.0, and that of chitinase activity was 4.0–5.0. The result of chitinase activity (▲) is from Ito et al. [8].

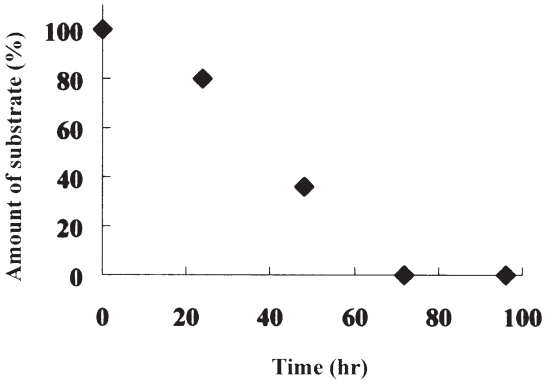

Figure 4. Isopeptidase activity assay with *T. japonica* lysozyme against ε-(γ-Glu)-L-Lys. Analysis of isopeptidase activity was performed with amino acid analysis. Values of activity indicate reduction in the amount of ε-(γ-Glu)-L-Lys.

Table 1. Inhibition with serine protease inhibitor (AEBSF).

	Isopeptidase activity (ΔOD405 after 4 h)		Lytic activity (ΔOD450/min) *M. luteus*	Chitinase activity (ΔOD420 after 1 h) glycol chitin
	L-γ-Glu-pNA	D-γ-Glu-pNA		
AEBSF (–)	0.15	0.31	0.33	86.2
AEBSF (+)	0.008	0.007	0.36	82.3

Isopeptidase activity toward L-γ-Glu-pNA or D-γ-Glu-pNA was measured at pH 7.0 and 37 °C. Lytic activity toward *M. luteus* was measured at pH 7.0 and 30 °C. Chitinase activity toward glycol chitin was measured at pH 5.5 and 40 °C.

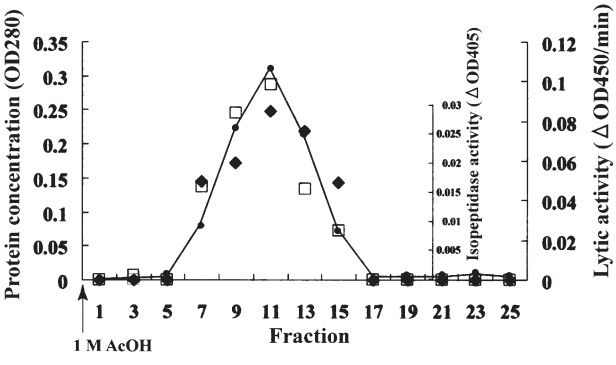

Figure 5. Profile of eluted protein from affinity column. The purified protein was loaded on an affinity column (chitin-coated celite). After washing with 150 ml of equilibration buffer, the column was eluted with 1 M acetic acid. Amount of protein is shown by the solid line. Lytic activity (□) and isopeptidase activity (◆) of each fractions is shown. Isopeptidase activity is indicated by ΔOD405 after 2 h.

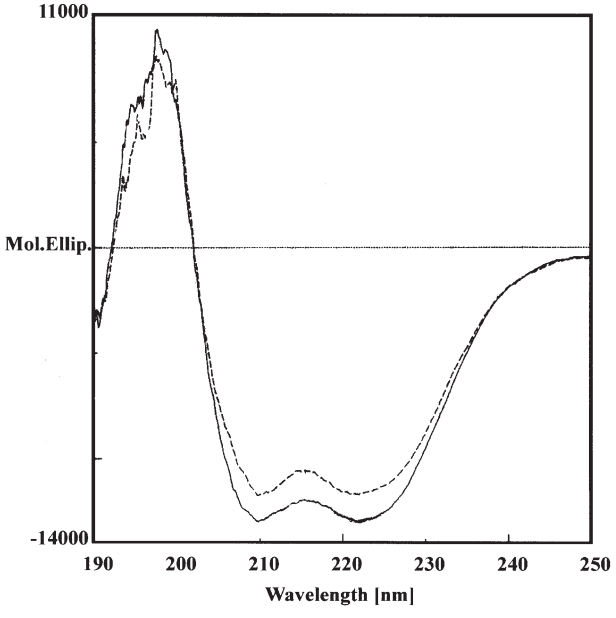

Figure 6. Secondary-structure analysis of *T. japonica* lysozyme using CD spectra. Solid line intact protein; dashed line, lyophilized protein.

treatment with AEBSF. This result suggested that *T. japonica* lysozyme has different sites for the two activities.

Assay of each enzyme activity for fractions eluted from an affinity column

T. japonica lysozyme has chitinase activity and thus the ability to bind chitin. The purified enzyme was applied to a chitin-coated celite column, washed with 0.1 M Tris-HCl containing 1.0 M NaCl and eluted by 1.0 M acetic acid (fig. 5). Then, fractions of 5 ml were collected, desalted, and concentrated tenfold. The concentration of protein in each fraction was determined with the Bicinchoninate (BCA) method, and lytic activity and isopeptidase activity were measured. Lytic activity was assayed using 20 µl of each fraction and 2 ml of *M. luteus* (0.35 mg/ml) suspension in 50 mM KH_2PO_4-NaOH buffer (pH 7). Isopeptidase activity was assayed using 200 µl of each fraction further concentrated five times and L-γ-Glu-pNA [0.25 mg/ml (1.75 mM)] solution in 0.05 M MOPS containing 0.01 M NaCl (pH 7.0). As a result, both activities were eluted simultaneously. This result confirmed that *Tapes japonica* lysozyme is a bifunctional enzyme possessing both lytic and isopeptidase activities.

Influence of lyophilization

Among three enzyme (lytic, chitinase, and isopeptidase activities) only the isopeptidase activity was largely degraded by lyophilization. The activity for L-γ-Glu-pNA was less than 55% and for D-γ-Glu-pNA less than 45%, while the lysozyme and chitinase activities were not affected. To investigate the influence of lyophilization, we measured CD spectra, but did not observe any significant change in the spectra (fig. 6).

Discussion

As previously reported by Ito et al. [8], *T. japonica* lysozyme possesses chitinase activity. The fact that *T. japonica* lysozyme hydrolyzed ε-(γ-Glu)-L-Lys showed that this lysozyme is also an isopeptidase. Isopeptidase activity for L-γ-Glu-pNA ($k_{cat}/K_m = 0.9\ s^{-1}\ M^{-1}$) of *T. japonica* lysozyme was less than that of destabilase ($1.6\ s^{-1}\ M^{-1}$) [20].

Originally, isopeptidase activity was very low even in destabilase and we worried that an impurity enzyme might be evoking this activity in our enzyme preparation. However, we could confirm that isopeptidase activity was absorbed on a chitin column and eluted simultaneously with lytic activity. This confirmed that *T. japonica* lysozyme possesses both chitinase and isopeptidase activity. The isopeptidase activity of *T. japonica* lysozyme was inhibited by AEBSF, but lytic and chitinase activities were not. The optimal pH for isopeptidase activity was 7.0 and that for chitinase activity was 5.0, clearly indicating that these activities are exhibited by independent active sites. Destabilase and chlamysin, which have 46% and 55% homology, respectively, to *T. japonica* lysozyme have given no indication so far of chitinase activity [7, 15]. On the basis of sequence homologies and characteristic activities, *T. japonica* lysozyme, destabilase, chlamysin and *Mytilus* lysozyme are homologous enzymes and could have a chitin-binding site.

We showed that isopeptidase activity was decreased by lyophilization, but lytic and chitinase activities were not. The result of CD spectra showed that little change was seen in the secondary structure by lyophilization. From these data, we presume that the active sites in the protein are separate. Detailed investigation of the influence of freeze-dry conditions using nuclear magnetic resonance and X-ray crystal structure analyses are underway. Since this lysozyme has isopeptidase activity against D-γ-Glu-pNA, we considered that it might enhance the lysozyme activity in harmony with chitinase activity. However, since AEBSF only inhibited the isopeptidase activity, this activity seems unlikely to be related to lytic activity. The isopeptidase activity might be efficiently utilized by *T. japonica* to digest bacteria in the late stage.

We feel that the genomic analysis of our enzyme is important as undertaken by Bachali et al. [9] and Nilsen and Myrnes [10]. We will attempt this, and so far have only analyzed and published the cDNA sequence of *T. japon-*

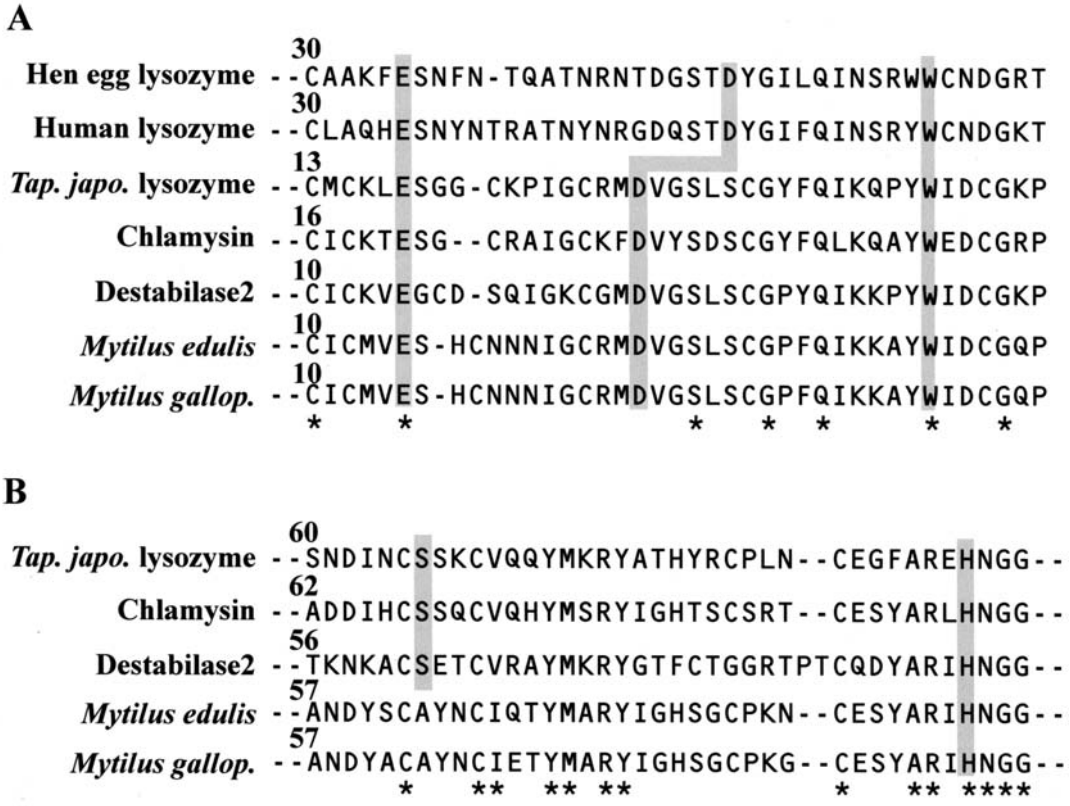

Figure 7. Multisequence alignment of *T. japonica* lysozyme and c-type lysozymes or its homologues. The amino acid sequence of two c-type lysozymes and three *T. japonica* lysozyme homologues were aligned using Clustal W [22]. Conserved amino acids are indicated by asterisks. Possible active residues are shadowed. (*A*) Amino acid sequences of *T. japonica* lysozyme [8], its homologues (destabilase 2: Genbank accession U24122, chlamysin; EMBL database accession number AJ250028; *Mytilus edulis*: AF334662; *M. galloprovincialis*: AF334665), and c-type lysozyme (EC 3.2.1.17) were aligned on the basis of regions of lytic activity. (*B*) Amino acid sequences of *T. japonica* lysozyme and its homologues were aligned on the basis of predicted catalytic residues (serine, histidine) as related to regions of isopeptidase activity [23].

ica lysozyme (AB091383). On the basis of studies by Bachali et al. [9] and Nilsen and Myrnes [10], the catalytic aspartate (D) in the c-type lysozymes is not conserved in i-type lysozyme. However, we have predicted that the catalytic aspartate in *T. japonica* lysozyme is D30 given the sequence homology with c-type lysozymes and pH dependence of chitinase activity (pK about 2.8). Moreover, this aspartate is conserved in i-type lysozymes. We think that the cause of the considerable difference in the position of the catalytic aspartate in i-type and c-type lysozymes is the existence of a cysteine within the homologous catalytic domain in i-type lysozymes.

It is interesting that the *T. japonica* lysozyme, a small protein, has both chitinase and isopeptidase activity, since bifunctional enzymes are usually large or complex proteins. Since the *T. japonica* lysozyme is a small, monomeric, and bifunctional enzyme, detailed analysis of its structure would be interesting (these studies are underway). Comparing the amino acid sequence of our enzyme with those of chicken and human lysozyme, we predicted that the region responsible for chitinase activity might be residues 18–46 of the *T. japonica* lysozyme (fig. 7). As catalytic residues for isopeptidase activity, H92, the only histidine residue present, and the conserved serine residue (S62) between destabilase 1 and 2 sequences in the second hydrophilic region were predicted by Zavalova et al. [23]. When we compare *T. japonica* lysozyme with destabilase, the H92 and S62 of destabilase are conserved in *T. japonica* lysozyme (H94 and S66). However, the serine is not conserved in the lysozyme sequence from *Mytilus edulis* and *Mytilus galloprovincialis.* Moreover, the lysozyme from *M. edulis* lacked isopeptidase activity [9]. Accordingly, we considered that catalytic residues for isopeptidase activity in *T. japonica* lysozyme are S66 and H94. Therefore, the region responsible for isopeptidase activity might be the residues 65–95. We suggest that the region for chitinase activity lies at the N-terminal end and that for isopeptidase activity at the C-terminal end of the *T. japonica* lysozyme sequence.

We must search for catalytic residues in detail using mutants of *T. japonica* lysozyme. We have already constructed an expression system for recombinant *T. japonica* lysozyme, and the bifunctional property has also been confirmed by gene engineering.

Thus, this enzyme is an interesting and unique lysozyme because it is a helix protein, judging from CD spectra. We assume that other i-type lysozymes have the same characteristics as *T. japonica* lysozyme.

Finally, thrombosis or fibrinogenolysis exhibited by destabilase has drawn great attention [17, 18]. *T. japonica* lysozyme is a small and very stable protein with isopeptidase activity, and we expect that it will have numerous applications.

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