Structural and Functional Properties of Chicken Lysozyme Fused Serine-rich Heptapeptides at the C-Terminus

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Two serine-rich heptapeptides, Ser-Ser-Ser-Lys-Ser-Ser-Ser (S6K) and Ser-Ser-Ser-Ser-Ser-Ser-Ser (S7), were fused to the C-terminus of chicken lysozyme (Lz) by genetic modification to improve the functional properties of lysozyme. The cDNAs of S6K-lysozyme (S6K-Lz) and S7-lysozyme (S7-Lz) were inserted into the expression vector of Pichia pastoris and secreted in yeast cultivation medium. The secretion amounts of S6K-Lz and S7-Lz were about 60% of that of wild-type lysozyme (Wt-Lz). The CD spectra showed that the conformation of S6K-Lz and S7-Lz was conserved regardless of the attachment of serine-rich peptides. The denaturation curves of S6K-Lz and S7-Lz also showed that the conformational changes were very small. The lytic activity of S6K-Lz and S7-Lz was almost the same as that of Wt-Lz, while the bactericidal activity against Escherichia coli of S6K-Lz and S7-Lz was greatly increased. The acetic acid-urea PAGE of phosphatase-treated S6K-Lz and S7-Lz indicated the possibility of phosphorylation of the fused serine-rich heptapeptides.

Key words: lysozyme; serine-rich heptapeptide; phosphorylation; Pichia pastoris

Hen egg lysozyme is a typical bactericidal protein. The bactericidal action is limited to Gram-positive bacteria. An enhancement of bactericidal activity against Gram-negative bacteria through chemical and genetic modifications of lysozyme has been reported.\(^1\)\(^\text{--}\)\(^4\) The covalent attachment of palmitic acid to lysyl residue,\(^1\) and the fusion of a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro)\(^2,3\) or polyproline chain\(^4\) at the C-terminus of lysozyme, have been effective in penetrating into the outer membrane of Gram-negative bacteria. The hydrophobic domains at the C-terminus are undoubtedly important for penetration into the microbial outer membrane alone, composed mainly of lipopolysaccharide. Although the fusion of a hydrophobic peptide at the C-terminus enhanced the bactericidal activity of lysozyme against Gram-negative bacteria, the effect of conformational change on the bactericidal activity of hydrophobic peptide-fused lysozyme is not fully understood. Laible and Germaine\(^5\) reported bactericidal action independent of lytic activity. Ibrahim et al.\(^6\) also reported that heat denaturation of lysozyme increased bactericidal activity against Gram-negative bacteria. It is well known that denaturation of proteins greatly increases surface hydrophobicity.\(^7\) Therefore, an increase in the hydrophobic domain of lysozyme must affect bactericidal action. In addition, it is possible that the insertion or attachment of peptides by genetic modification of proteins causes a change in the conformation of proteins. In order to elucidate further the molecular mechanism of modified lysozyme, we constructed lysozyme-fused hydrophilic peptides at the C-terminus to investigate structural and functional properties. Two serine-rich heptapeptides, Ser-Ser-Ser-Ser-Ser-Ser-Ser (S6K) and Ser-Ser-Ser-Ser-Ser-Ser-Ser (S7), with highly conserved repeat motifs in phosvitin and with the potential to be phosphorylated, were used as hydrophilic peptides. Interestingly, the bactericidal action of the S6K and S7 lysozymes was greatly improved in a mode similar to that of hydrophobic peptide-attached lysozyme. The possibility of phosphorylation in serine-rich heptapeptides was also proposed.

Materials and Methods

Materials. Micrococcus lysodeikticus (Micrococcus l.ysodeikticus) cells were purchased from Sigma (St. Louis, Missouri, U.S.A.). E. coli K-12 IFO 3301 (lacO lacZ lacY) was obtained from the Institute for Fermentation, (Osaka, Japan). All other chemicals were of analytical grade for biochemical use.

Construction of Expression Plasmids of Serine-rich Heptapeptide-fused Lysozymes. The recombinant plasmid pKK-1 containing a full-length hen lysozyme cDNA was used, and serine-rich heptapeptide-fused lysozyme cDNAs were prepared by the PCR method as follows. The sequence of the sense primer was 5’-GG-
GCTCGAGAAAAAGAAAAATCTTTTGGACGATGGAGC-3', containing an Xho I site (underline). The sequences of the anti-sense primer for S6K-lysozyme (S6K-Lz) and 57-lysozyme (S7-Lz) were 5'-TCTAGACTCAGGTCCTGGATCAGGCTGCTGGCACCCGGAGGCTTCTGACGCTC-TGATC-3' and 5'-TCTAGACTCAGGTCCTGGATCAGGCTGCTGGCACCCGGAGGCTTCTGACGCTC-TGATC-3', respectively, containing an Xho I site (underline), a stop codon, and a serine-rich heptapeptide sequence (bold). PCR was performed with ExTaq polymerase in a GeneAmp® PCR system 2400 using standard PCR conditions with a hot start at 94 °C for 5 min, 25 cycles of 30 sec of denaturation at 94 °C, 30 sec of annealing at 59 °C, and 4 min of elongation at 72 °C. The amplification products were purified by electrophoresis on a 1.0% low-melting agarose gel and subcloned into pT7 Blue T-vector (Novagen). The DNA sequencing was performed on a 1.0% low-melting agarose gel and 5% stacking gel, containing 0.1% SDS. Electrophoresis was carried out at a constant current of 20 mA for 72 h with shaking. The far-ultraviolet (200–260 nm) circular dichroism spectra were produced according to the method of Kato and Takaki. Samples were adjusted to 0.04 mg/ml in 50 mM glycine–HCl buffer (pH 3.5). CD spectra were recorded at 25 °C on a J-600 spectropolarimeter (Jas, Tokyo, Japan) with a 1.0 cm cuvette. CD spectra were represented in terms of mean residue ellipticity (degrees·cm²·dmol⁻¹). Thermal denaturation of lysozymes was measured by monitoring the change in the ellipticity of the CD spectra at 222 nm with a temperature increase from 55 °C to 85 °C at a heating rate of 1 °C/min. The apparent unfolding fraction (Fapp) was represented as a function of temperature to the denaturation curves.

Acetic Acid-Urea PAGE, Acetic acid-Urea PAGE was carried out using a microslab gel electrophoresis system. The running gel was 20% polyacrylamide, 0.9 M acetic acid, and 2.5 M urea, and the stacking gel was 7.5% polyacrylamide, 0.375 M potassium acetate (pH 4.0), and 2.5 M urea. Electrophoresis was carried out at a constant voltage of 100 V in 0.9 M acetic acid.

Measurement of Lytic Activity. The lytic activity of lysozyme was determined according to the turbidimetric method by a lysis of Micrococcus lysodeikticus (M. lysodeikticus) cells. The lyophilized M. lysodeikticus cells were suspended in 100 mM citrate–phosphate buffer (pH 3.0–7.0) or sodium phosphate buffer (pH 8.0). A 100 μl aliquot of lysozyme solution (40 μg/ml) was added into a 2.4 ml cell suspension (170 μg/ml). The initial decrease in absorbance at 450 nm caused by lysis of M. lysodeikticus cells was measured at 20 °C for 1 min with a Hitachi U-2000 spectrophotometer. Activity was expressed as a percentage of that observed for the wild type at pH 6.0.

Measurement of Bactericidal Activity. Gram-negative bacteria, E. coli K-12, were cultured for 10 h in LB medium, harvested, and washed 3 times with sterilized water, then suspended in 50 mM citrate-phosphate buffer (pH 6.0). 100 μl of the suspension (10⁴ cells/ml) was mixed with 100 μl of lysozyme solution (final concentration 0.05% w/v). The mixture was incubated at 25 °C for 0, 10, 20, and 30 min, and then 50 μl was surface plated onto MacConkey agar plates. All plates were incubated at 37 °C overnight. The percent survival was represented as a percentage to the colony number from a control solution without lysozymes.

Circular Dichroism (CD) Analysis. The N-terminal amino acid sequence of the lysozymes was determined by automatic Edman degradation using protein sequencer PPSQ-21A (Shimadzu, Japan) coupled to an on-line high-performance liquid chromatography SPD-10A (Shimadzu, Japan). The lysozymes were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Ltd., UK) using a standard SDS-PAGE method.
semi-dry blotting apparatus (Atto, Tokyo, Japan). The transferred proteins were visualized by Ponceau S staining, then the excised band was subjected to Edman degradation to sequence.

Phosphatase Treatment. Phosphorylation analysis was performed using acid phosphatase (Sigma). 20 μl of each sample solution (0.5 mg/ml, 90 mM citrate buffer, pH 4.8) was mixed with or without (control) 0.1 unit of acid phosphatase and incubated at 37°C overnight. Then the same volume of native sample buffer was added and subjected 10 μl to Acetic acid Urea-PAGE.

Results

Expression and purification of serine-rich heptapeptide-fused lysozymes

The S6K-Lz and S7-Lz were secreted in the culture medium and purified with a CM-Toyopearl 650M column (Tosoh, Japan). As shown in Fig. 1, the elution pattern of S6K-Lz (panel A) is almost the same as that of S7-Lz (panel B). Both elution patterns showed a small peak and large peak. These peaks were collected and subjected to SDS-PAGE. As shown in Fig. 2, the first peak represents a high molecular weight band of lysozyme.

Fig. 1. Elution Patterns of S6K Lysozyme (A) and S7 Lysozyme (B) on a CM-Toyopearl 650M Column.
The supernatants of yeast culture medium were diluted with distilled water 5 times and applied to a CM-Toyopearl 650M column. The adsorbed lysozyme was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM sodium phosphate buffer (pH 8.0) and monitored at 280 nm.

Fig. 2. SDS-PAGE Patterns of S6K- and S7-Lysozymes.
The gel sheet was stained with Coomassie brilliant blue R250.
Lane M, molecular weight marker (phosphorylase 97.4 kDa, bovine serum albumin 66.3 kDa, aldolase 42.4 kDa, carbonic anhydrase 30.0 kDa, trypsin inhibitor 20.1 kDa, lysozyme 14.4 kDa); Lane 1, Wt-Lz; Lane 2, S6K-Lz (low molecular weight); Lane 3, S6K-Lz (high molecular weight); Lane 4, S7-Lz (low molecular weight); Lane 5, S7-Lz (high molecular weight).
S6K and S7-Lzs (lanes 3 and 5) and the main peak represents a lower molecular weight band of S6K and S7-Lzs (lanes 2 and 4). The N-terminal amino acid sequence of these components was analyzed. The sequence of the high molecular weight component contains 9 residues of α-factor signal peptide (Glu-Glu-Gly-Val-Ser-Leu-Glu-Lys-Arg) incorrectly cleaved by endoproteases kex2, while the low molecular weight component was correctly cleaved to have the correct N-terminal of lysozyme. The presence of an additional signal peptide of α-factor have been reported for H5-lysozyme and glycosylated lysozyme secreted in *P. pastoris*. The secretion amounts of S6K-Lz and S7-Lz were 7.2 mg/l and 6.8 mg/l, respectively. The secretion amount was calculated in the purified form and three times higher than that of hydrophobic pentapeptide (H5) fused-lysozyme.

**Lytic activity of serine-rich heptapeptide-fused lysozymes**

The lytic activity of lysozymes was measured from the degree of lysis of *Micrococcus lysodeikticus* cells. As shown in Fig. 3, the lytic activity of S6K-Lz and S7-Lz was slightly lower than that of Wt-Lz in the range of pH 3.0–8.0. Under the optimal pH condition (pH 6.0), the maximal lytic activity of S6K-Lz and S7-Lz was about 92% of that of Wt-Lz. This suggests that the fusion of hydrophilic peptides (S6K and S7) has a slight effect on the lytic activity of lysozyme. It is probable that the affinity of modified lysozymes with the substrate may be slightly affected by attached serine-rich peptides.

**Bactericidal activity of serine-rich heptapeptide-fused lysozymes**

The bactericidal activity of lysozymes against *E. coli* K-12 is shown in Fig. 4. S6K-Lz and S7-Lz greatly decreased the survival ratio of *E. coli* K-12 during incubation time, while Wt-Lz did not. This result indicated that potent bactericidal action against Gram-negative bacteria had been endowed by the attachment of serine-rich peptides at the C-terminus of lysozyme. The results of the lytic activity and bactericidal action of S6K- and S7-Lz are compared with H5 in Table 1. As shown there, S6K- and S7-Lz showed bactericidal action comparable to that of H5-Lz, although the action was not as strong as that of H5-Lz.

**Conformational stability of serine-rich heptapeptide-fused lysozymes**

Conformational change in S6K-Lz and S7-Lz was investigated using far-UV CD. As shown in Fig. 5, the ellipticity of S6K-Lz and S7-Lz at the range of wavelengths 200–260 nm. Liu et al. reported that the fusion of hydrophobic pentapeptide (H5) at the C-terminus of lysozyme decreased the electrostatic interactions between Lys13 in the first α-helix (residues 5 to 15) and the C-terminal carboxyl group, thereby decreasing the stability of the ordered structure of the α-helix in lysozyme. But, the hydrophilic heptapeptides (S6K and S7) had less effect on the electrostatic interactions compared to hydrophobic pentapeptide-fusion lysozyme (H5-Lz).

**Table 1.** Secretion Amount, Enzymatic Activity and Bactericidal Action of Wild, S6K, S7, and H5 Lysozymes Secreted from *Pichia pastoris*

<table>
<thead>
<tr>
<th>Lysozyme</th>
<th>Secretion amount (mg/l)</th>
<th>Enzymatic activity (%)</th>
<th>Bactericidal action (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>12.3</td>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td>S6K-Lz</td>
<td>7.2</td>
<td>93</td>
<td>20</td>
</tr>
<tr>
<td>S7-Lz</td>
<td>6.8</td>
<td>92</td>
<td>10</td>
</tr>
<tr>
<td>H5-Lz*</td>
<td>2.2</td>
<td>80</td>
<td>5</td>
</tr>
</tbody>
</table>

* a) data from references 12 and 2. b) survival ratio of *E. coli* K12 after incubation at 25 °C for 30 min in the presence of various lysozymes (5 μg/ml).
mational stability was estimated from thermal denaturation curves, which were determined by a change in ellipticity at 222 nm during heating from 55 to 85 °C (Fig. 6). The mid-transition temperature (T_m) of S6K-Lz and S7-Lz was calculated from the thermal denaturation curve, indicating slightly lower values, 76.3 °C and 76.0 °C respectively, than the 76.6 °C of Wt-Lz. This confirms that the conformational stability of S6K-Lz and S7-Lz decreased slightly.

Phosphorylation of serine-rich heptapeptide-fused lysozymes

Phosphorylation of S6K-Lz and S7-Lz by post-translational modification in P. pastoris was investigated. As shown in Fig. 7, the bands of S6K-Lz and S7-Lz moved more slowly than that of wild lysozyme, while the bands of their peptide-fusion lysozymes treated with acid phosphatase moved faster than that of the control in acetic acid-urea PAGE. This suggests that the negative charges were increased in S6K-Lz and S7-Lz by the phosphorylation of serine residues. They may be phosphorylated during secretion in P. pastoris. It is probable that one or two residues of serine were phosphorylated.

Dephosphorylation treatment of S6K-Lz and S7-Lz with phosphatase had no effect on lytic activity or bactericidal activity, suggesting that the phosphorylation of serine-rich peptide-fused lysozyme is not involved in the enhancement of bactericidal action.

Discussion

An attempt was made to attach serine-rich peptides, highly phosphorylated motifs in phosvitin, to C-terminus of lysozyme. The hydrophilic heptapeptides S6K and S7 were successfully fused to the C-terminus of lysozyme and secreted in P. pastoris. Although the lytic activities of S6K-Lz and S7-Lz were slightly decreased, they showed strong bactericidal action in a manner similar to hydrophobic pentapeptide-fused lysozyme (H5-Lz).2,3) The bactericidal action of H5-Lz is considerably stronger than that of S6K-Lz or S7-Lz.2) It appears likely that the attachment of hydrophobic peptide in H5-Lz causes the conformational changes as observed in the CD spectrum.2) The attachment of hydrophilic peptides (S6K and S7) might also cause the conformational changes as judged by the decreases in the Tm, although the decrease was smaller than that of H5-Lz. It has been reported by Ibrahim et al.14) that a helix-loop-helix peptide of lysozyme confers potent bactericidal activity with membrane permeabilization action. The lytic activity is important for the antimicrobial action against Gram-negative bacteria.15,16) Besides the lytic activity, the bactericidal activity of S6K-Lz and S7-Lz may be caused by a small change in the conformation that facilitates the access of the antimicrobial peptide in...
lysozyme to the microbial organism. The dephosphorylation of S6K-Lz and S7-Lz was confirmed by the decrease in negative charges with phosphatase treatment. It is probable that one or two phosphate residues attach to serine-rich peptides, because phosphokinase may be specific to serine residues. A phosphorylated β-casein secreted in P. pastoris has been reported by Choi et al.\textsuperscript{17} The phosphorylation of serine-rich peptides described in this paper supports that yeast P. pastoris has a phosphorylation system as well as mammalian cells. The effect of phosphorylation in the attached serine-rich peptides on bactericidal action was considered. The bactericidal action of S6K and S7-Lzs did not, however, change after phosphatase treatment. Therefore, the phosphorylation of serine-rich peptides does not affect bactericidal action, but conformational change does affect it.

References


