Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function

Hisham R. Ibrahim*, Tetsuji Matsuzaki, Takayoshi Aoki

Department of Biochemistry and Biotechnology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

Received 28 July 2001; accepted 24 August 2001

First published online 7 September 2001

Edited by Pierre Jolles

Abstract A catalytically inactive mutant of hen egg white lysozyme was constructed by site-directed mutagenesis to elucidate the role of enzymatic activity on its antimicrobial activity against Gram-positive bacteria. The catalytic residue aspartic acid at position 52 of lysozyme was substituted with serine (D52S-Lz) and the mutant cDNA was inserted into a yeast expression vector, pYES-2. Western blot analysis indicated that the mutation did not affect secretion of the D52S-Lz lysozyme into the medium of the expressing *Saccharomyces cerevisiae*, INVSC1. In addition, circular dichroism and fluorescence spectral analysis revealed no change in the structure of D52S-Lz compared to that of wild-type (Wt-Lz) lysozyme. The mutation (D52S) abolished the catalytic activity of lysozyme. Antimicrobial tests against *Staphylococcus aureus* and *Bacillus subtilis* revealed that the catalytically inactive D52S-Lz was as bactericidal as the Wt-Lz lysozyme. Heat treatment leading to enzyme inactivation had no effect on the bactericidal activity of either wild-type or the mutant D52S-Lz lysozyme. The binding affinity of D52S-Lz to the isolated peptidoglycan of *S. aureus* was unaffected. Our results provide the first demonstration of direct genetic evidence that the antimicrobial activity of lysozyme is operationally independent of its muramidase activity, and strongly suggest the antimicrobial action of lysozyme is due to structural factors.

Key words: Lysozyme; Antibacterial activity; Site-directed mutagenesis; D52S lysozyme; Yeast expression; Peptidoglycan binding

1. Introduction

Lysozyme is known as a powerful antibacterial protein widely distributed in various biological fluids and tissues including avian egg, plant, bacteria, and animal secretions, tears, saliva, milk, respiratory and cervical secretions, and secreted by polymorphonuclear leukocytes [1]. It belongs to a class of enzymes that lyses the cell walls of certain Gram-positive bacteria, as it splits the bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of the peptidoglycan (PG) in the bacterial cell walls. Beside antimicrobial activity, lysozyme has many other functions, including inactivation of certain viruses [2], important roles in surveillance of membranes of mammalian cells [3], it enhances phagocytic activity of polymorphonuclear leukocytes and macrophages [4,5] and stimulates proliferation and antitumor functions of monocytes [6].

Although conclusive evidence is lacking, it has long been believed that the antimicrobial action of lysozyme would merely be attributed to its catalytic function on bacterial cell walls. The importance of lysozyme as an active defense molecule has been debated, since in most bacteria the PG layer is not directly accessible to this enzyme. Under physiological conditions only a minority of Gram-positive bacteria are susceptible to lysozyme, and it has been suggested that the main role of lysozyme is to participate in the removal of bacterial cell walls after the bacteria have been killed by antimicrobial polypeptides present in egg albumin, insect hemolymph [7] or by complement in animal serum [8]. This is in line with the notion that the lytic action of lysozyme does not kill susceptible bacteria under physiological conditions, osmotically balanced [9]. The literature abounds with evidence pertaining to the functional role of lysozyme in various tissues as a defense mechanism, suggesting that it may primarily be related to structural properties and secondarily to its direct bacteriolytic action [10,11]. Recently, we found that heat denaturation of lysozyme progressively inactivates the enzyme while greatly promoting its antimicrobial action to Gram-negative bacteria. Surprisingly, denatured lysozyme devoid of enzyme activity exhibited bactericidal activity against Gram-positive bacteria, suggesting an action independent of catalytic function [12–16]. However, it was hard to obtain concrete evidence that muramidase activity makes no contribution in lysozyme's antimicrobial activity against Gram-positive bacteria because of the complex structural changes accompanying thermal inactivation. It was impossible to discern if the observed antimicrobial activity is unique to the denatured lysozyme or it is a general action regardless of structural changes of lysozyme.

In this study, we decided to discriminate between the native and non-native antimicrobial action of lysozyme and to verify more precisely the role of muramidase activity in the inherent antibacterial action of lysozyme. For this, site-directed mutagenesis was employed to inactivate the enzyme by substituting one of its two catalytic residues, Asp, with Ser at position 52. Aspartic acid 52 was selected for this investigation because mutation of this residue has been reported to abolish muramidase activity without affecting binding affinity of mutant

*Corresponding author. Fax: (81)-99-285 8525.
E-mail address: hishamri@chem.agri.kagoshima-u.ac.jp (H.R. Ibrahim).

Abbreviations: NLz, authentic lysozyme from hen egg white; Wt-Lz, wild-type lysozyme expressed in yeast; D52S-Lz, mutant lysozyme (Asp-52-Ser); CFU, colony-forming unit; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; BSA, bovine serum albumin; PG, peptidoglycan

0014-5793/01/$20.00
PII: S0014-5793(01)02872-1

lysozyme to substrate or inhibitor [17–19]. Thus, Asp-52-Ser mutation would be expected to produce inactive lysozyme while restoring its native structure as well as substrate binding ability. We explore here, for the first time, that the genetically inactive lysozyme is a potent bactericide against typical Gram-positive bacteria regardless of its enzyme activity.

2. Materials and methods

2.1. Materials

Micrococcus lysodeikticus, the microbial substrate of lysozyme, was purchased from Sigma (St. Louis, MO, USA). Oligodeoxyribonucleotides of the mismatching primer (5′-GAGTACCAGCTACGGAAT-3′) for mutagenesis was synthesized by Takara Shuzo (Kyoto, Japan). PG from *Staphylococcus aureus* IFO 14462 was isolated according to the hot SDS method [20]. Mouse anti-lysozyme monoclonal IgG was provided by Dr. Y. Yamaguchi (Fukuyama University, Japan). All other chemicals were of the highest grade commercially available.

2.2. Bacterial strains and plasmids

Bacterial strains used were *Escherichia coli* TOP10F′ (F′(lacI^{q}Tn*10*(Tet^R))mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG), used as a host cell in DNA manipulation, and *Saccharomyces cerevisiae* INVSC1 (MATα, his3-D1, leu2, trp1-289, ura3-52), an expression yeast. The pYES-2 plasmid, a yeast expression vector, was purchased from Invitrogen (San Diego, CA, USA). *Staphylococcus aureus* IFO 14462, *Bacillus subtilis* IFO 3007 and *Bacillus cereus* IFO 13690 were obtained from the Institute of Fermentation (Osaka, Japan) as test microorganisms for antimicrobial assays. M13 mp19 bacteriophage was from Takara Shuzo. The pKK1 plasmid, which contains the full length of the pre-lysozyme cDNA, was provided by Dr. I. Kumagai, University of Tokyo, Japan.

2.3. Site-directed mutagenesis and construction of expression plasmid

The small *Eco*RI-*Hin*dIII fragment of pKK1 [21], containing the full length of pre-lysozyme cDNA (Wt-Lz), was inserted into M13 mp19 and the single stranded plasmid was used for site-directed mutagenesis. The mutant lysozyme cDNA (D52S-Lz), having the catalytic aspartic acid 52 residue substituted with serine, was constructed by using the mutagenic oligonucleotide primer, 5′-GAGTACCAGC-TACGGAAT-3′, with the Sculptor in vitro mutagenesis system (RPN 1526) (Amersham, UK). The mutation was confirmed by DNA sequencing. The small *Eco*RI-*Sph*I fragment of the full length of mutant or wild-type cDNAs was ligated with the large *Eco*RI-*Sph*I fragment of the pYES-2 expression vector downstream of the GAL1 promoter and upstream of the CYC1 transcription terminator. The expression plasmids of the wild-type and mutant lysozyme were termed pYES-Wt and pYES-D52S, respectively.

2.4. Expression of mutant lysozyme in yeast

The yeast expression vectors pYES-Wt and pYES-D52S were transformed to *S. cerevisiae* INVSC1 according to the lithium acetate method [22]. The transformants were selected on ura$^-$ yeast minimal medium (YMM) agar plates. The transformant yeasts were inoculated into 10 ml of YMM-ura$^-$ (containing 0.1% glucose and 5% glycerol) and incubated for 24 h at 29°C with shaking. This pre-culture was then transferred to 1 l of the same medium in a 3 l flask at 29°C with shaking. When the absorbance (A_{600}) of the culture reached 0.3–0.5, expression was induced by adding the appropriate amount of a concentrated stock solution of (filter-sterilized) galactose to a final concentration of 2%. Shaking was continued for 8–10 h at 29°C.

2.5. Purification of mutant D52S lysozyme

The induced culture of *S. cerevisiae* INVSC1 was centrifuged at 5000×*g* for 10 min at 4°C. The supernatant was diluted four times with deionized water and applied to a CM-Toyopearl 650 column (1.5×5.0 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5), and after washing protein was eluted with the same buffer containing 0.5 M NaCl. Fractions containing lysozyme were pooled, diluted 10 times with the same buffer and rechromatographed on a CM-Toyopearl 650 column (1.5×10 cm). The lysozyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. The fractions (3 ml) containing lysozyme were collected and dialyzed in a Spectra/por dialysis tube (M_r 6000–8000 Da cut-off) against distilled water. The peaks were vacuum-concentrated in a SpeedVac centrifuge. Protein concentration of the secreted lysozymes was estimated from the digital image of the SDS–PAGE gel, under reducing conditions, by Electrophoretic Documentation Analysis System 120 (EDAS 120) equipped with a DC120 Camera and Kodak ID 2.02 image analysis software (Eastman Kodak Co., New York, NY, USA), using the band intensity of authentic hen egg white lysozyme as a standard.

2.6. Muramidase activity assay

The lysis of *M. lysodeikticus* cells was determined according to the turbidimetric method, previously reported [13], based on the decrease in turbidity of a 1.9 ml cell suspension (170 μg dry cells/ml) in 50 mM potassium phosphate buffer (pH 6.2) following the addition of a 100 μl portion of the pooled fractions or the purified lysozyme solution (20–160 μg/ml distilled water) after equilibration to achieve constant absorbance (0.75–0.8). For the pH activity profile 50 mM buffer of Na-acetate (pH 4.0–6.0) or K-phosphate (pH 6.0–8.5) was used. The decrease in absorbance at 450 nm (25°C) was monitored using a Hitachi U-2000 recording spectrophotometer. The activity is expressed as the rate of decrease in absorbance per minute.

2.7. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE, under reducing conditions (5% β-mercaptoethanol), was performed using a 5% acrylamide stacking gel and 15% separating gel containing 0.1% SDS according to the method of Laemmli [23]. Protein bands were visualized by Coomassie brilliant blue R-250 (CBB).

2.8. Immunoblotting

Immunoblot analysis of the recombinant lysozyme (Wt-Lz and D52S-Lz) was performed after resolving the protein bands on SDS–PAGE (5–15% acrylamide gel) as described above. The protein bands in the gel were electroblotted onto a polyvinylidene difluoride (PVDF) membrane by a semidry unit at 0.8 mA/cm^2 for 30 min, using the Towbin buffer system [24]. The blots were blocked by 2% bovine serum albumin (BSA), and then immunostained using mouse anti-lysozyme monoclonal IgG and secondary goat anti-mouse IgG alkaline phosphatase-conjugated antibody.

2.9. Antibacterial assay

The liquid broth method [25] was used to assess the bactericidal activity of the recombinant proteins. Mid-logarithmic phase cells, grown in BHI broth, were washed and resuspended (to give 2×10^6 cells/ml) in a buffered Bacto-peptone broth (pH 7.4). Each bacterial suspension (100 μl) was mixed with an equal volume of the same medium containing the test lysozyme (final concentration 50 μg/ml). Controls were incubated in the absence of protein. The mixture was incubated at 30°C for 1 h, then a 30 μl portion or dilutions (in 0.85% saline) were spotted onto nutrient agar plates. The colony-forming units (CFU) were obtained after incubating the agar plates at 37°C for 18 h. All assays were performed in triplicate and the results, unless otherwise noted, are the means of three independent experiments.

2.10. PG binding assay by enzyme-linked immunosorbent assay

Microtiter plates (96 wells) were coated with purified PG of *S. aureus* by incubating 100 μl/well (10 μg/ml in 0.1 M Na_2CO_3, pH 9.6) at 37°C for 3 h. After rinsing and blocking with BSA, lysozyme sample was allowed to interact for 1 h at 37°C, and then flicked out. Mouse anti-lysozyme IgG monoclonal IgG was incubated with the plates for 1 h, and then was substituted with the alkaline phosphatase-conjugated goat anti-mouse IgG for 1 h. A 100 μl aliquot of 0.1% of *p*-nitrophenylphosphate-Na_2 in diethanolamine buffer (pH 9.8) was added and incubated at 25°C until sufficient color had developed. The reaction was stopped by addition of 50 μl/well of 2 N NaOH, and absorbance at 405 nm was measured in a microtiter plate reader. Mean absorbance and S.E.M. were calculated after subtracting the value of controls from four replications of each well.

3. Results

It was deduced from a detailed analysis of the complex

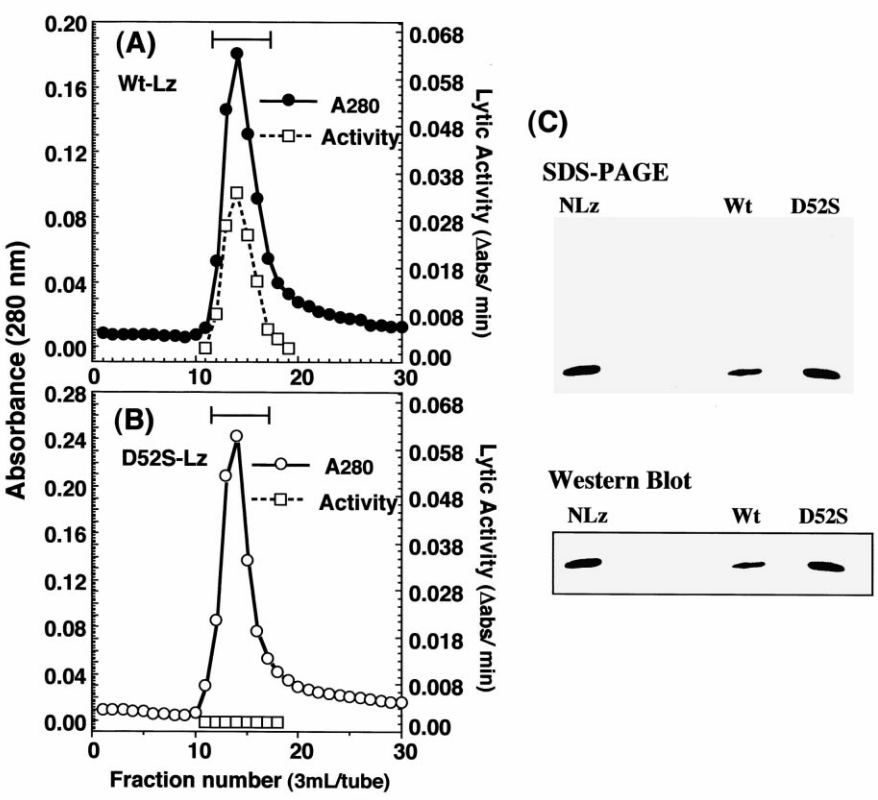

Fig. 1. Purification of Wt-Lz (A) and D52S-Lz (B) lysozymes from yeast medium on a CM-Toyopearl 650 column. The transformant *S. cerevisiae* INVSC1, harboring vectors pYES-Wt or pYES-D52S, grown to late log phase ($A_{600} \approx 0.5$) in YMM-ura$^-$, then expression was induced for 10 h at 29°C by the addition of galactose (2%). The cell-free supernatants were applied to a CM-Toyopearl 650 column, then eluted with a linear gradient of 0–0.5 M NaCl in 50 mM Tris–HCl buffer (pH 7.5). The absorbance was monitored at 280 nm (circles), and a portion from each fraction was checked for muramidase activity against *M. lysodeikticus* (open squares). C: Protein peaks were dialyzed, vacuum-concentrated and applied to reducing SDS–PAGE and protein bands were visualized with CBB (top) or electroblotted to a PVDF membrane and immunostained using mouse anti-NLz monoclonal IgG and alkaline phosphatase-conjugated anti-mouse IgG goat antibody (bottom).

between lysozyme and hexamer of *N*-acetylglucosamine by X-ray crystallography that Glu-35 and Asp-52 are the catalytic residues [26]. The mutation of Glu-35 to Asp or Asp-52 to Glu in hen [27] or human [28] lysozyme caused inactivation of the enzyme. Glu-35 participates in catalysis in protonated form and Asp-52 does so in dissociated form. A covalent complex between mutant Asp-52-Ser lysozyme and substrate oligosaccharides was detected by electrospray mass spectrometry [19], however, because there is no system to remove the sugar intermediate the hydrolysis was improbable [18]. Accordingly, in order to keep the molecular dynamics as well as the binding ability of the inactive mutant lysozyme to PG unchanged we chose the substitution of Asp-52 by serine that will be the most appropriate for the purpose of this investigation.

3.1. Expression and purification of D52S-Lz

Upon induction of expression with galactose, a considerable amount of the mutant D52S-Lz and Wt-Lz lysozyme was secreted in the culture medium of yeast. Both recombinant lysozymes were eluted, with a linear gradient of 0–0.5 M NaCl, from the CM-Toyopearl 650 column at the same position (Fig. 1). The peak of D52S-Lz did not show muramidase activity, assessed by using *M. lysodeikticus* cell walls as substrate, in any fraction (Fig. 1B). To confirm that the secreted mutant protein, which has no enzyme activity, is lysozyme, the purified peaks were run on SDS–PAGE under reducing

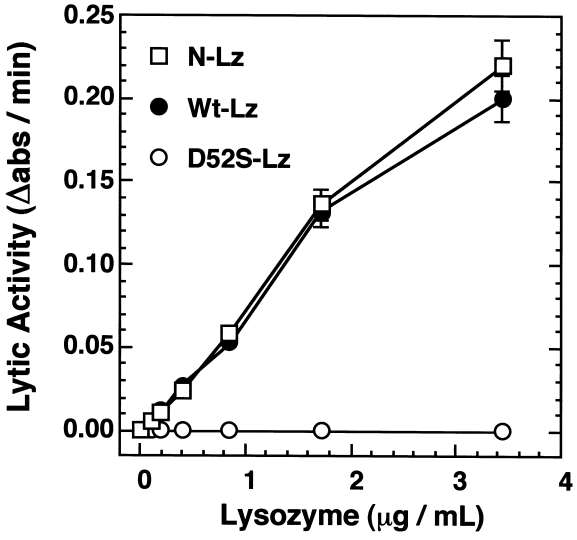

Fig. 2. Muramidase activity of the purified Wt-Lz and D52S-Lz lysozymes from INVSC1 yeast medium on a CM-Toyopearl 650 column. The decrease of turbidity at 450 nm of *M. lysodeikticus* cell suspension was monitored in 50 mM K-phosphate buffer (pH 6.2), containing various concentrations of enzyme. Activity is expressed as the rate of change in absorbance per minute.

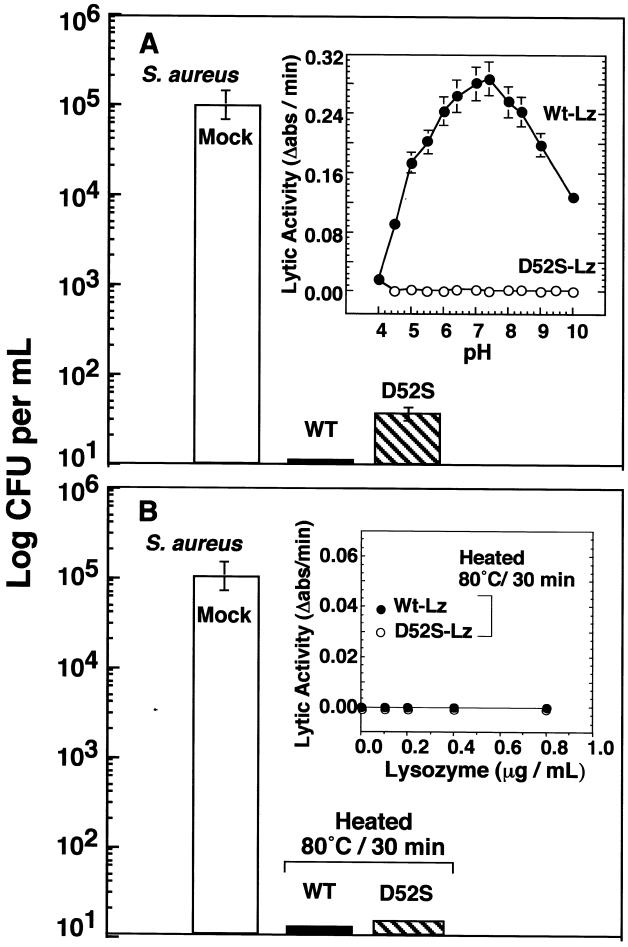

Fig. 3. Anti-staphylococcal activity of Wt-Lz and D52S-Lz lysozymes. A: The bactericidal assay was performed by incubating *S. aureus* with 50 μg/ml protein at 30°C for 1 h, then dilutions were spotted onto nutrient agar plates. The CFU were obtained after incubating the plates at 37°C for 18 h. Inset: pH–activity profile of Wt-Lz and D52S-Lz (final concentration 3.4 μg lysozyme/ml) as described in Section 2. B: Wt-Lz and D52S-Lz were heat-denatured in 10 mM Na-phosphate buffer (pH 6.0) and tested for anti-staphylococcal activity. Inset: Muramidase activity of Wt-Lz and D52S-Lz upon heat denaturation at different protein concentrations.

conditions and analyzed on immunoblots stained with specific monoclonal antibody to hen egg white lysozyme (Fig. 1C). Protein staining of the gel (Fig. 1C, top) indicated that the mutant D52S-Lz has electrophoretic mobility similar to that of Wt-Lz and authentic hen egg white lysozyme (NLz). Immunostaining of the blot (Fig. 1C, bottom) identified the mutant D52S-Lz as lysozyme.

3.2. Enzymatic activity of D52S-Lz

Fig. 2 shows the muramidase activities of authentic Lz, Wt-Lz and D52S-Lz lysozymes for *M. lysodeikticus* cells as a function of lysozyme concentration at pH 6.2. Within the linear range of lysozyme concentrations (final concentration 0.2–1.8 μg/ml), the lytic activity of Wt-Lz is almost the same as that of NLz. However, D52S-Lz showed no enzymatic activity at any protein concentration tested. The mutant D52S-Lz was inactive even after extending the reaction time up to 30 min (data not shown). It should be noted that similar results of activity were obtained when the enzymatic reaction was carried out at 37°C (data not shown). The results confirm that the mutation of Asp-52-Ser abolishes the enzymatic activity of lysozyme at neutral pH. Prior to investigating the antibacterial activity of the muramidase-inactive mutant lysozyme we examined the conformational changes that might occur with this mutation (Asp-52-Ser). We found that this mutation did not alter either the secondary or the tertiary structure of lysozyme as judged by circular dichroism (CD) and intrinsic fluorescence emission spectra (data not shown). This demonstrated that the muramidase-inactive lysozyme (D52S-Lz) restores the same conformation as that of the Wt-Lz and agrees with the reported crystal structure of the mutant D52S hen egg white lysozyme [17].

3.3. Antibacterial activity to Gram-positive bacteria

Fig. 3 shows the bactericidal action of Wt-Lz and D52S-Lz against *S. aureus* cells. Both Wt-Lz and the mutant D52S-Lz lysozymes were bactericidal to this strain with approximately 4 and 3.6 log reduction in cell viability, respectively (Fig. 3A). It was confirmed from the pH dependence of the hydrolytic activity profile, within the range of pH 4–10, that the mutant Asp-52-Ser is catalytically inactive, while Wt-Lz showed the common bell-shaped pH–activity profile (Fig. 3A, inset).

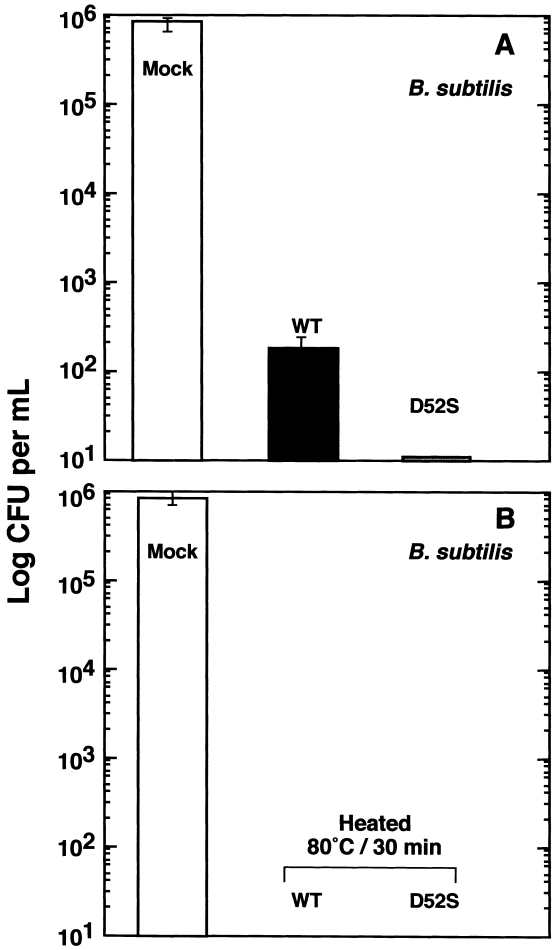

Fig. 4. Antibacterial activity against *B. subtilis* of Wt-Lz and D52S-Lz lysozymes. A: The bactericidal assay was performed as described for *S. aureus* in the legend of Fig. 3A. B: Antibacterial activity against *B. subtilis* of heat-denatured Wt-Lz and D52S-Lz as described for *S. aureus* in the legend of Fig. 3B.

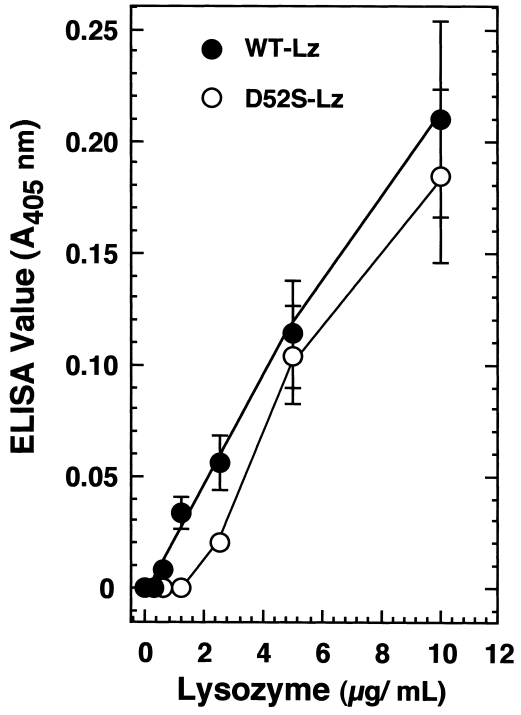

Fig. 5. Solid-phase binding of Wt-Lz and D52S-Lz to the isolated PG of *S. aureus*. Wells were coated with PG then blocked with BSA. Various concentrations of Wt-Lz (solid circles) or D52S-Lz (open circles) were added. The amount of bound lysozyme was quantitated by incubation with mouse anti-lysozyme IgG monoclonal IgG then the alkaline phosphatase-conjugated goat anti-mouse IgG and developed with *o*-nitrophenyl-β-D-galactoside. Each data point is the mean of triplicates.

Wild-type lysozyme exhibited the maximum lytic activity at pH 7.4, the pH at which the antibacterial assay was performed. From the results of Fig. 3A, it is apparent therefore that hydrolysis of PG in the cell walls and the killing of bacteria by lysozyme are two independent processes. This was further confirmed by testing the antibacterial activity upon heat-induced inactivation of both constructs (Fig. 3B). The heat-inactivated Wt-Lz and D52S-Lz exhibited the same antibacterial potency against *S. aureus* regardless of residual enzyme activity (Fig. 3B, inset). Furthermore, both constructs Wt-Lz and D52S-Lz showed a remarkable in vitro bactericidal activity against two other Gram-positive strains, *B. subtilis* (Fig. 4) and *B. cereus* (data not shown; identical to the data in Fig. 4). Interestingly, the enzymatically inactive D52S-Lz exhibited stronger activity, more than one log of killing, against *B. subtilis* than Wt-Lz (Fig. 4A). However, upon heat-induced inactivation both D52S-Lz and Wt-Lz exerted a comparably strong bactericidal activity against *B. subtilis* (Fig. 4B) and also *B. cereus* (data not shown).

3.4. Binding capacity to isolated PG of S. aureus

Lysozyme interacts with Gram-positive bacteria by initially binding to PG of the cell walls. We investigated the interaction between the mutant D52S-Lz and isolated PG from *S. aureus*. Both D52S-Lz and Wt-Lz were found to exhibit essentially similar binding affinity to PG (Fig. 5). At low concentrations the binding of Wt-Lz to PG was slightly more pronounced than D52S-Lz. This might be ascribed to the previous observation that the (*N*-acetylglucosamine)$_4$–D52S complex portrays a distorted, sofa conformation for subsite D [17]. However, the results indicate that mutation did not unfavorably alter the binding to the bacterial cell walls.

4. Discussion

Although structural and physiological [29,30] as well as enzymatic [1,28] characteristics of lysozyme have been clearly elucidated, its structure–bactericidal relationship has yet to be fully understood. Recently, we suggested that the antimicrobial action of lysozyme is related to conformational changes and seems to be decoupled from its enzyme activity. The study involved irreversible heat denaturation at different pHs [12,13,15,16]. However, because heat-denatured lysozyme has a non-native conformation, it is still not certain that the enzymatic activity of lysozyme makes a contribution to the antibacterial action, particularly against the lysozyme-sensitive Gram-positive bacteria.

The results of this study provide the first demonstration of direct genetic evidence that the principal bactericidal action of lysozyme against the Gram-positive bacteria *S. aureus* and *B. subtilis* was independent of its catalytic function (Figs. 3 and 4). It is of general interest that the mutant D52S-Lz has a fairly stronger bactericidal activity against *B. subtilis* than Wt-Lz (Fig. 4A). Since both Wt-Lz and D52S-Lz have the same antibacterial potency when heat-denatured (Figs. 3B and 4B), the results support the conclusion that the killing of these typical Gram-positive bacteria would be ascribed to structural features of the lysozyme molecule rather than enzymatic bacteriolysis. However, monitoring the absorbance (at 600 nm) of *B. subtilis* cell suspensions in the presence of the muramidase-inactive mutant (D52S-Lz) revealed considerable bacterial lysis (data not shown). This striking datum thus indicates that the killing of Gram-positive bacteria by lysozyme is basically a bacteriolytic action but operationally independent of its muramidase activity. Given the fact that both Wt-Lz and D52S-Lz have almost the same efficiency in binding bacterial PG (Fig. 5), the killing of these typical Gram-positive bacteria involves interaction with the bacterial cell walls. Most likely the initial attraction between the positively charged lysozyme and the negatively charged bacterial surface (PG) seems to be based on electrostatic forces.

It is of interest to note that the potency of Wt-Lz and D52S-Lz against *B. subtilis* differed (Fig. 4A). Because the structure of D52S-Lz was indistinguishable from that of Wt-Lz, as revealed by CD and intrinsic fluorescence emission spectra, it is likely that the difference in their antibacterial response against *B. subtilis* would be ascribed to the interference of muramidase activity of Wt-Lz to the structure-related action (Fig. 4A). These results seem contradictory, but apparently the main mode of bactericidal action against Gram-positive bacteria involves the existence of some microbe-associated target of lysozyme, other than direct hydrolysis of PG. This observation is in agreement with the note that bacterial autolysins play an essential role in expression of the bactericidal activity of lysozyme against oral microflora [11]. It has also been reported that cell death of *B. subtilis* caused by surfactants at low concentrations results from induced cell autolysis [31,32]. These observations together with the results of the present work suggest that autolysins are involved in killing and lysis of bacteria by lysozyme that may actually be induced by intrinsic structural motifs other than murami-

dase activity. The exploration of the ability of D52S-Lz to trigger such non-enzymatic mechanisms of microbicidal action would merit further investigation.

Lysozyme has been recognized as an important factor in the body's defense against bacterial [4,33–36] and HIV-1 [37] infections as well as the lysis of tumor cells [6,38–40]. The tantalizing question, therefore, is, what is the role muramidase activity plays in lysozyme's defense functions? Simply, lysozyme appears to behave like a biological response modifier that elicits host responses by interacting with pathogens or intestinal bacteria, thus liberating PGs of high and low molecular weight that become the agents responsible for the specific immune cell activation. The reported immunostimulatory activity of lysozyme [3,6] is in support of this conclusion. The elucidation of the catalytically independent mechanisms of bactericidal action of D52S-Lz as well as other biological functions reported for lysozyme is now in progress.

In summary, the data provide the first direct genetic evidence that the bactericidal action of lysozyme is operationally independent of its catalytic function. The results suggest two main modes of catalytically independent bactericidal action of lysozyme: an indirect bacteriolytic-dependent mode (such as that against *B. subtilis* and *B. cereus*) and a lysis-independent death mode (such as that against *S. aureus*). We suggest that the lysis-dependent mode involves regulation of cell wall-associated autolysins through a potential molecule that could bind lysozyme and thus regulate autolytic activities. However, cell death without lysis is likely to result from impaired membrane functions through the binding of a certain domain of lysozyme with the bacterial surface. Eventually, the genetic evidence presented in this study will provide a clearer understanding of the mechanism by which lysozyme kills bacteria and the rules that govern its potency may provide guidance in drug design.

Acknowledgements: This work was supported by a Scientific Research Grant (C-2 #13660129) from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] Jolles, J. and Jolles, P. (1984) Mol. Cell. Biochem. 63, 165–189.
[2] Hasselberger, F.X. (1978) in: Uses of Enzymes and Immobilized Enzymes, p. 128, Nelson-Hall, Chicago, IL.
[3] Osserman, E.F., Klockars, M., Halper, J. and Fischer, R.E. (1974) in: Lysozyme (Osserman, E.F., Canfield, R.E. and Beychok, S., Eds.), Academic Press, New York.
[4] Kokoshis, P.L., Williams, D.L., Cook, J.A. and Di-Luzio, N.R. (1978) Science 199, 1340–1342.
[5] Thakur, A., Chauhan, A. and Willcox, M.D. (1999) Aust. N.Z. J. Ophthalmol. 27, 224–227.
[6] Lemarbre, P., Rinehart, J.J., Kay, N.E., Vesella, R. and Jacobs, H.S. (1981) Blood 58, 994–999.
[7] Boman, H.G., Faye, I., Gudmundsson, G.H., Lee, J.Y. and Lidholm, D.A. (1991) Eur. J. Biochem. 201, 23–31.
[8] Flescher, E., Keisari, Y., Lengy, J. and Gold, D. (1991) Parasitology 103, 161–164.
[9] Brooks, G.F., Butel, J.S. and Ornston, L.N. (1991) in: Medical Microbiology, pp. 20–22, Prentice-Hall, London.
[10] Cisani, G., Varaldo, P.E., Pompei, R., Valisena, S. and Satta, G. (1989) Microbios 59, 73–83.
[11] Laible, N.J. and Germaine, G.R. (1985) Infect. Immun. 48, 720–728.
[12] Ibrahim, H.R., Higashiguchi, S., Juneja, L.R., Kim, M. and Yamamoto, T. (1996) J. Agric. Food Chem. 44, 1416–1423.
[13] Ibrahim, H.R., Higashiguchi, S., Koketsu, M., Juneja, L.R., Kim, M., Yamamoto, T., Sugimoto, Y. and Aoki, T. (1996) J. Agric. Food Chem. 44, 3799–3806.
[14] Ibrahim, H.R., Higashiguchi, S., Sugimoto, Y. and Aoki, T. (1996) Food Res. Int. 29, 771–777.
[15] Ibrahim, H.R., Higashiguchi, S., Sugimoto, Y. and Aoki, T. (1997) J. Agric. Food Chem. 45, 89–94.
[16] Ibrahim, H.R. (1998) Nahrung/Food 42, 187–193.
[17] Hadfield, A.T. et al. (1994) J. Mol. Biol. 243, 856–872.
[18] Ito, Y., Ogata, Y., Hashimoto, Y., Kuroki, R. and Imoto, T. (1995) Protein Eng. 8, 962–967.
[19] Lumb, K.J. et al. (1992) FEBS Lett. 296, 153–157.
[20] Russell, R.R. (1979) J. Gen. Microbiol. 114, 109–115.
[21] Ibrahim, H.R., Yamada, M., Matsushita, K., Kobayashi, K. and Kato, A. (1994) J. Biol. Chem. 269, 5059–5063.
[22] Ito, H.Y., Fukuda, K., Murata, K. and Kimura, A. (1992) J. Bacteriol. 153, 163–168.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Gravel, P., Golaz, O., Walzer, C., Hochstrasser, D.F., Turler, H. and Balant, L.P. (1994) Anal. Biochem. 221, 66–71.
[25] Ibrahim, H.R., Sugimoto, Y. and Aoki, T. (2000) Biochim. Biophys. Acta 1523, 196–205.
[26] Phillips, D.C. (1966) Sci. Am. 215, 78–90.
[27] Imoto, T. (1990) in: Protein Engineering (Ikehara, M., Ed.), pp. 153–158, Japan Scientific Societies Press and Springer-Verlag, Tokyo.
[28] Muraki, M., Harata, K., Hayashi, Y., Machida, M. and Jigami, Y. (1991) Biochim. Biophys. Acta 1079, 229–237.
[29] Blake, C.C., Koeing, D.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1965) Nature 206, 757–761.
[30] Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C. and Rupley, J.A. (1972) in: The Enzymes, Vol. 7, pp. 665–868, Academic Press, New York.
[31] Cho, H.Y., Tsuchido, T., Ono, H. and Takano, M. (1990) J. Ferment. Bioeng. 70, 11–14.
[32] Tsuchido, T., Svarachorn, A., Soga, H. and Takano, M. (1990) Antimicrob. Agents Chemother. 34, 781–785.
[33] Akinbi, H.T., Epaud, R., Bhatt, H. and Weaver, T.E. (2000) J. Immunol. 165, 5760–5766.
[34] Cole, A.M., Dewan, P. and Ganz, T. (1999) Infect. Immun. 67, 3267–3275.
[35] Travis, S.M., Conway, B.A., Zabner, J., Smith, J.J., Anderson, N.N., Singh, P.K., Greenberg, E.P. and Welsh, M.J. (1999) Am. J. Respir. Cell Mol. Biol. 20, 872–879.
[36] Wang, C. and Shelef, L.A. (1991) J. Food Sci. 56, 1251–1254.
[37] Lee-Huang, S., Huang, P.L., Sun, Y., Kung, H.F., Blithe, D.L. and Chen, H.C. (1999) Proc. Natl. Acad. Sci. USA 96, 2678–2681.
[38] Sava, G., Perissin, L. and Zorzet, S. (1988) Clin. Exp. Metast. 6, 245–253.
[39] Sava, G., Ceschia, V. and Pacor, S. (1989) Anticancer Res. 9, 1175–1180.
[40] Scott, M.G. and Hancock, R.E. (2000) Crit. Rev. Immunol. 20, 407–431.