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A Mutation Study of Catalytic Residue Asp 52 in Hen Egg Lysozyme

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We constructed a system for the expression and secretion of mature hen lysozyme by yeast using an intermediate "secretion-signal cassette" vector, pKP1700, containing the yeast invertase signal sequence and an expression vector, pAM82, for secretion and maturation of the enzyme. Using this system, mutants of hen lysozyme were produced and the catalytic mechanism in hen lysozyme was definitely confirmed. The hydrolytic activity of D52A as to substrate $(NAG)_6$ at pH 5.0 was obviously decreased to one-four hundredth of that of the wild type. The acidic limb of the pH-activity profile observed for the wild-type was not observed for D52A, and the pK_a of Glu 35 on the alkaline limb was seen for both enzymes. Moreover, no structural change was detected on X-ray analysis of D52A. Therefore, we confirmed that dissociated Asp 52 assists catalysis by producing an electrostatic field and by stabilizing the oxocarbonium ion intermediate in the dissociated form.

Key words: catalytic mechanism, expression system, hen lysozyme, mutagenesis, X-ray analysis.

In the field of protein engineering, it is necessary to obtain target proteins. The advanced gene manipulation methodology has made it possible to express heterologous genes in various hosts such as *Escherichia coli*, yeast, and animal cells, and to obtain their gene products in large amounts. We succeeded in producing chicken lysozyme by using an *E. coli* expression system in the previous study (*1*). However, as lysozyme was obtained as inactive and insoluble inclusion bodies, a renaturation step was necessary to obtain an active enzyme. Renaturation of lysozyme was not always easy; moreover, the lysozyme produced using the *E. coli* expression system contained an extra methionine residue at the NH_2-terminus. In contrast, using the yeast, *Saccharomyces cerevisiae*, it has been reported that a number of eucaryotic proteins including human and hen lysozymes were synthesized as active forms with the correct NH_2-termini (*2-8*).

Hen egg lysozyme is a carbohydrate hydrolase that catalyzes the hydrolysis of β-1,4 glycosidic bonds of polysaccharides of such as a homopolymer of *N*-acetyl-D glucosamine (chitin), and an alternating copolymer of *N*-acetyl-D glucosamine (NAG) and *N*-acetylmuramic acid (NAM), which is the major constituent of bacterial cell walls (*9*). Binding sub-sites for six monosaccharides were recognized in the active site cleft and called A to F. Based on the X-ray crystallographic data, it was proposed that the cleavage occurred between the sugars located at sub-sites D and E through the cooperative reaction of Glu 35 and Asp 52 (*10*). Namely, Glu 35 acts as a general-acid catalyst to protonate the glycosidic oxygen, while Asp 52, as a negative charge, stabilizes the oxocarbonium ion intermediate. The involvement of Asp 52 in the catalysis was also shown by the mutation of Asp to Asn (*11, 12*) or Ser (*13*). However, as residual activity was observed with these mutations, the precise role of Asp 52 in the catalysis is still a matter of debate (*13-15*). Moreover, structural analyses of these mutants has not been accomplished yet. In particular, when we observe a drastic decrease in activity, the structural integrity of the mutant must be precisely investigated.

In this study, we constructed a system for the expression and secretion of mature hen lysozyme in the yeast, *S. cerevisiae*, using an intermediate "secretion-signal cassette" vector, pKP1700, containing the yeast invertase signal sequence, and an expression vector, pAM82, for secretion and maturation of the enzyme. Also, we confirmed the catalytic mechanism of hen lysozyme by analyzing the catalysis of mutant lysozymes as to Asp 52, and X-ray crystallographic analysis of D52A.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, and T4 DNA ligase were purchased from Takara Shuzo (Kyoto), or Toyobo (Osaka). CM-Toyopearl 650M, a cation-exchange resin for the purification of secreted lysozymes, was obtained from Tosoh (Tokyo). *Micrococcus luteus*, a substrate of lysozyme was purchased from Sigma Chemical (St. Louis). ABI Dye Primer Cycle Sequencing Kits for DNA sequencing were purchased from Applied Biosystems Japan (Tokyo). The other chemicals used were of analytical or biochemical grade.

Strains, Plasmids, and Phage—*E. coli* RR1 [*supE44, hsdS20* (r_B^-, m_B^-), *ara-14, proA2, lacY1, galK2, rpsL20* (Sm^r), *xyl-5, mlt-1*] (*16*), and JM110 [*dam, dcm, supE44, hsdR17, thi, leu, rpsL, lacY, galK, galT, ara, tonA, thr,*

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Abbreviations: D52A, mutant lysozyme with Asp 52 mutated to Ala; D52C, mutant lysozyme with Asp 52 mutated to Cys; D52N, mutant lysozyme with Asp 52 mutated to Asn; D52S, mutant lysozyme with Asp 52 mutated to Ser; D52T, mutant lysozyme with Asp 52 mutated to Thr; $(NAG)_n$, β-1,4-linked *n*-mer of *N*-acetyl-D glucosamine.

tsx, Δ (*lac-proAB*)/F′ [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*Δ-M15]] (*17*) were used for cloning with plasmid and M13 phage, respectively. *S. cerevisiae* strain AH22 [*α*, *leu2*, *his4*, *can1*, *cir*$^+$) (*18*), which was kindly supplied by Dr. F. Hamada (Chemo-Sero-Therapeutic Research Institute, Kumamoto), was used for the expression and secretion of hen lysozyme. *E. coli* cells were grown in LB broth as described previously (*19*). Yeast cells were grown in a modified Burkholder minimal medium (*20*) supplemented with 8% sucrose, 5 g/liter asparagine, 100 mg/liter histidine, 100 mg/liter tryptophan and 0.6 mM KH_2PO_4 (pH 5.3).

Plasmid pKLZ 58, which contains hen lysozyme cDNA in a cloning vector pUC9 derivative, was the previous construct (*21*). Plasmid pAM 82 (*22*), which was also kindly supplied by Dr. F. Hamada, was used as a vector for the expression of hen lysozyme in yeast. Phagemid pUC118 (*23*) and bacteriophage M13 mp18 (*17*) were used for the construction of pKP1700.

DNA Manipulation—The procedures for purification of DNA, restriction enzyme digestion, ligation, agarose-gel electrophoresis, and transformation of competent *E. coli* cells were as described previously (*24, 25*). DNA sequencing was carried out with an Applied Biosystems DNA sequencer 373A and a Dye Primer Cycle Sequencing Kit (Applied Biosystems). Site-directed mutagenesis was performed by the method of Kunkel *et al.* (*26*).

Activity of Mutant Lysozymes—To 700 μl of a Blue-*Micrococcus luteus* suspension (1.6 mg/ml) pre-incubated in 0.1 M phosphate buffer (pH 7.0) containing 0.5 M sucrose, 100 μl of a lysozyme solution ($A_{280}=0.1$) was added, and then the mixture was incubated at 40°C for 15 h. The reaction was stopped by the addition of 50 μl of 1 N NaOH. After centrifugation, the absorbance of the supernatant at 600 nm was measured (*27*).

$(NAG)_6$ (5×10^{-4} M) and each mutant lysozyme (2.4×10^{-7} M) were incubated at pH 5.0 and 40°C for 72 h. Under the same conditions, the wild type was incubated for 4 h. Gel filtration HPLC for analysis of the digests was performed on a Cellulofine column, which was eluted with 0.1% HCl at the flow rate of 0.4 ml/min and 55°C. Since the decrease in the peak of $(NAG)_6$ obeyed first-order kinetics, the rate constant for the cleavage of $(NAG)_6$ by lysozyme was calculated by plotting the logarithm of the peak of $(NAG)_6$ against the incubation time.

Spectroscopic Titration of Glu 35—The pH dependence of the tryptophyl fluorescence of the wild-type or D52A lysozyme in 0.1 M KCl at 30°C in the presence of $(NAG)_3$ was measured with a Hitachi F-2000 fluorescence spectrophotometer as described (*28*) by measuring the fluorescence at 330 nm with excitation at 280 nm. The concentrations of the protein and $(NAG)_3$ employed were 2.4×10^{-6} and 1.0×10^{-4} M, respectively.

X-Ray Analysis—Crystallization was carried out using a vapor diffusion technique at pH 4.7. Intensity data collection for the wild-type and D52A was carried out with an automated oscillation camera system, R-AXIS IIC (RIGAKU), equipped with an Imaging Plate detector, on a Cu rotating anode generator operated at 40 keV and 100 mA at room temperature. The R_{merge} values of the wild-type and D52A based on the intensities were 5.0 and 4.5%, respectively. The completeness of the reflections at 1.95 Å resolution of the wild type and D52A was 92.1 and 96.7%, respectively [$F>1\sigma(F)$]. The *R*-factors of the wild-type and D52A were 16.6 and 16.3%, respectively. Refinement of the structures was carried out by use of both programs TURBO-FRODO and X-PLOR installed on SGI Indigo.

RESULTS

Construction of "Secretion-Signal Cassette" Vector pKP1700 for Yeast Expression—A Secretion-signal cassette vector, pKP1700, containing the *S. cerevisiae* invertase signal sequence (*29*), was derived from bacteriophage M13 mp19 DNA (Fig. 1). Two synthetic oligonucleotides, Oligo-1 and Oligo-2 (Table I), that carry the yeast invertase signal sequence, were inserted between the *Sal*I and *Nsi*I sites of pKP1697 to yield pKP1698. pKP1697 is a M13 phage vector constructed by replacing the multi-cloning site of M13 mp18 with two synthetic oligonucleotides, Oligo-3 and -4 (Table I), carrying *Sal*I, *Nsi*I, *Eco*RI, *Hin*dIII, and *Xho*I sites in its sequence. Then, with two synthetic oligonucleotides, Oligo-5 and -6 (Table I), the *Xho*I site of pKP1698 was replaced with a *Sal*I site, yielding pKP1699. A DNA fragment with a multi-cloning site, *Eco*RI-*Sac*I-*Kpn*I-*Sma*I-*Bam*HI-*Xba*I-*Xho*I-*Pst*I-*Sph*I-*Hin*dIII of pKP1696, derived from phagemid pUC118, was then inserted between the *Eco*RI and *Hin*dIII sites of pKP1699. The resulting phage vector, pKP1700, carries a *Sal*I fragment with the yeast invertase signal sequence upstream from the multi-cloning site.

Construction of a Plasmid Producing the Wild-Type Lysozyme—An expression plasmid, pKLZ100, containing a chicken lysozyme gene with the invertase signal sequence at its NH_2-terminal end was constructed using pKP1700, as follows. The *Eco*RI-*Hin*dIII fragment of pKLZ58 (*21*)

TABLE I. **Synthetic oligonucleotides for the construction of the vector and mutagenesis.**

Oligo-1 & Oligo-2:
5′-TCGACGTATATGATGCTTTTGCAAGCCTTCCTTTTCCTTTTGGCTGGTTTTGCAGCTAAGATATCTGCA-3′
3′-GCATATACTACGAAAACGTTCGGAAGGAAAAGGAAAACCGACCAAAACGTCGATTCTATAG-5′
M L L E A F L F L L A G F A A K I S A
Oligo-3: 5′-AGCTCTCGAGAAGCTTGTCGAATTCCATGCATAGTCGAC-3′
Oligo-4: 3′-GAGCTCTTCGAACAGCTTAAGGTACGTATCAGCTGTTAA-5′
*Xho*I *Hin*dIII *Eco*RI *Nsi*I *Sal*I
Oligo-5: 5′-AGCTTTTGTCGACA-3′
Oligo-6: 3′-AAACAGCTGTAGCT-5′
*Hin*dIII *Sal*I
Oligo-7: 5′-GGGAGTACCTGTTACGGAATCC-3′ (D52C)
Oligo-8: 5′-GGGAGTACCTCTTACGGAATCC-3′ (D52S)
Oligo-9: 5′-GGGAGTACCGCTTACGGAATCC-3′ (D52A)
Oligo-10: 5′-GGGAGTACCACCTACGGAATCC-3′ (D52T)

carrying lysozyme cDNA with an ATG (Met) codon just upstream from the first codon, AAA (Lys), of the mature lysozyme was inserted between the *Eco*RI and *Hin*dIII sites of the secretion-signal cassette vector, pKP1700. The frame between the invertase signal sequence and that of the lysozyme of the resulting recombinant phage vector was adjusted by site-directed mutagenesis using oligonucleotide Oligo-7 as a primer, yielding pKLZ99. The *Sal*I fragment of pKLZ99 harboring the invertase-lysozyme fusion gene was then transferred to the *Xho*I site located just downstream from the *PHO5* promoter of the yeast expression vector, pAM82. The resulting plasmid was designated as pKLZ100, which cannot be cut by either *Xho*I or *Sal*I anymore. Transformation of *S. cerevisiae* AH22 was performed according to the spheroplast method of Hinnen *et al.* (*18*). Since yeast AH22 requires histidine and leucine, and pAM82 contains the leu 2 gene, yeast clones harboring pAM82 derivatives were selected on a modified Burkholder minimal medium plate containing only histidine as an amino acid.

Construction of Plasmids Producing Mutant Lysozymes—Mutant lysozyme genes in which Asp 52 is mutated to Cys (D52C), Ser (D52S), Ala (D52A), and Thr (D52T) were prepared by use of pKLZ99 as a template by site-directed mutagenesis. The structure of the mutagenic primer used to replace Asp 52 with Cys, Ser, Ala, and Thr is shown in Table I. The mutation in the lysozyme was confirmed by DNA sequencing. Cloning to expression vector pAM 82 and transformation of *S. cerevisiae* AH22 were performed as above.

Expression of the Wild-Type and Mutant Lysozymes as to Asp 52 from Yeast—Expression and secretion of chicken lysozyme were carried out by growing AH22 cells harboring pKLZ100 in a low phosphate synthetic medium (0.6 mM KH_2PO_4), since the *PHO5* promoter of pAM82 is under the control of phosphate in the medium. pAM82 is a *S. cerevisiae-E. coli* shuttle vector harboring the replication origins of 2μ plasmid and pBR322, and the *PHO5* promoter to express foreign genes inserted into the *Xho*I site immediately downstream from the promoter. The kinetics of the lysozyme production are shown in Fig. 2. Under the conditions employed, the increase in turbidity ceased around 50 h, presumably because of depletion of phosphate in the medium. Coinciding with this cessation of growth, synthesis of the lysozyme began and continued for 70–100 h. The amount of lysozyme secreted into the culture medium was about 4 mg/liter at the plateau. The lysozyme secreted into the medium was purified and characterized as described (*28*). Namely, the lysozyme secreted into the culture medium was purified by cation-exchange chromatography. The lysozyme produced by yeast cells had the same properties as the hen egg-white lysozyme as to

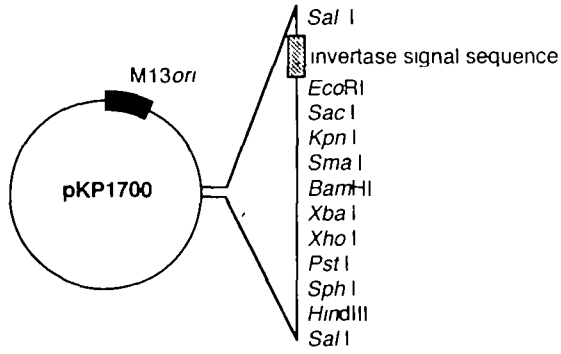

Fig. 1. **An intermediate vector, pKP1700, for construction of the invertase signal sequence fusion.** The M13 origin of DNA replication is depicted by the filled segment. The yeast invertase signal sequence is indicated by the hatched segment. The multicloning site was introduced downstream from the signal sequence.

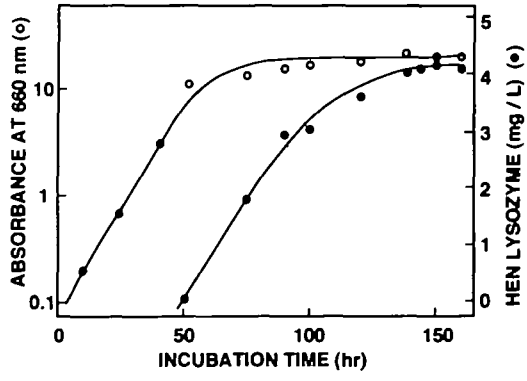

Fig. 2. **Growth curve and the amount of secreted lysozyme on the cultivation of cells harboring the wild type lysozyme expressing plasmid pKLZ100.** Open and closed circles show the absorbance at 660 nm and the amount of secreted lysozyme estimated from its activity in the culture medium, respectively. After the yeast cells had grown in the high-P_i Burkholder medium, the production of lysozyme was induced by omitting P_i (*22*). The activity was measured using a Blue-*Micrococcus luteus* suspension as a substrate, and native chicken lysozyme as a control (*27*). The details are given in the text.

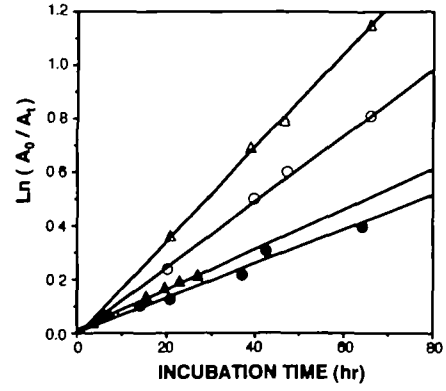

Fig. 3. **Hydrolytic activity toward $(NAG)_6$ of Asp 52 mutant lysozymes.** $(NAG)_6$ (5×10^{-4} M) in 0.04 M acetate buffer (pH 5.0) was incubated with lysozyme (2×10^{-7} M) at 40°C. The decrease in $(NAG)_6$ was measured as described in the text. Open triangles, open circles, filled triangles, and filled circles show D52S, D52C, D52T, and D52A, respectively. A_0/A_t is the ratio of the peaks of $(NAG)_6$ on incubation for 0 and t hours. The details are given in the text.

TABLE II. **Activity of the wild-type and Asp 52 mutant lysozymes.**

Lysozyme	Blue-*M. luteus* lytic activity (%)	Hydrolytic rate as to $(NAG)_6$ (h^{-1})
Wild-Type	100	50.4±2.7
D52A	1.50±0.005	0.12±0.02
D52S	0.71±0.02	0.32±0.004
D52T	0.91±0.04	0.12±0.02
D52C	0.62±0.02	0.19±0.04

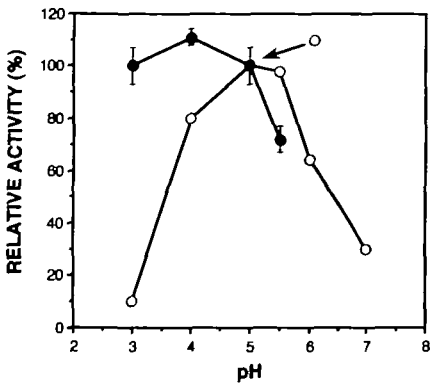

Fig. 4. **pH profile of hydrolytic activity toward $(NAG)_6$ of the wild type and D52A lysozymes at 40°C.** Open and filled circles show the wild type and D52A, respectively. The D52A and wild type lysozymes were incubated with $(NAG)_6$ at pH 5.0 and 40°C for 72 and 4 h, respectively. Gel filtration HPLC for analysis of the digests was performed on a Cellulofine column. The details are given in the text.

activity-measurement, analysis of the N-terminal amino acid sequence and amino acid composition, peptide-mapping of the tryptic peptides of the secreted lysozyme, SDS-PAGE and native-PAGE (data not shown).

Expression and secretion of mutant lysozymes as mature forms from yeast were carried out in the same way for the wild-type. Confirmation of mutations was carried out by means of DNA sequence and amino acid composition analysis and peptide-mapping of each tryptic peptide of a reduced-*S*-carboxymethylated mutant lysozyme on reversed phase-HPLC (data not shown). The yields of D52A, D52T, D52S, and D52C secreted into the medium were 0.50, 0.44, 0.95, and 0.073 mg/liter, respectively. Probably, the stability of mutants reflects the yield.

Catalysis of Mutant Lysozymes as to Asp 52—In order to determine the precise activity of lysozyme, we measured the rate of cleavage of $(NAG)_6$ into $(NAG)_4$ and $(NAG)_2$ by lysozyme at pH 5.0 and 40°C (Fig. 3). Since, in these mutants, there was no lag phase in the time course of the decrease in the amount of $(NAG)_6$, the predominant presence of a stable intermediate, which was suggested in D52S by a previous study (*6*), may be excluded. The hydrolytic activity values for $(NAG)_6$ of D52A, D52T, D52S, and D52C are shown in Table II. From the biological aspect, the lytic activities of these mutants against Blue-*M. luteus*, which is also a sensitive method for evaluating activity (*10*), were determined. These values are also shown in Table II. Clearly, on the mutation of Asp 52 to Ala, Thr, Cys, or Ser, the activity decreased drastically (0.2-1.5%), but was evidently retained to some extent.

pH Dependence of the Activity of D52A—It is interesting that D52A, in which Ala cannot form hydrogen bonding, exhibited similar activity to the other mutants, D52T, D52C, D52S, and D52N, in which the mutated residues are potentially capable of hydrogen bonding. Therefore, we focused our attention on D52A and examined its pH-activity profile. The relative activities as to those at pH 5.0 of the wild-type and D52A at various pHs are shown in Fig. 4. The wild-type showed a bell-shaped pH-activity profile with an optimum at pH 5. In the case of D52A, the acidic limb of the pH-activity profile had disappeared and its

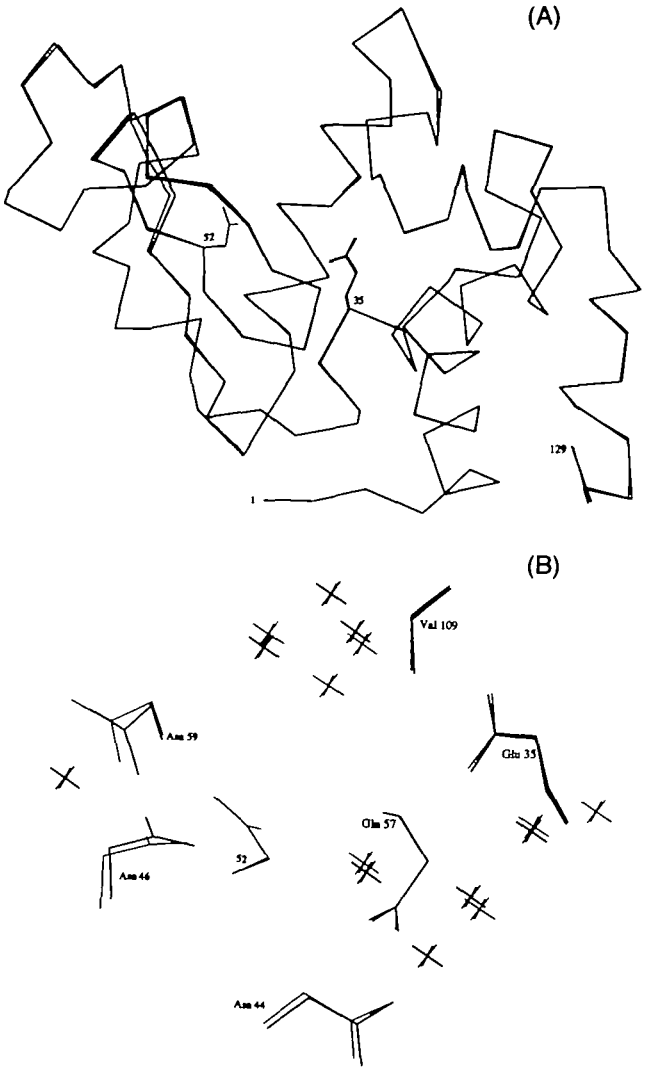

Fig. 5. **Structural comparison of the D52A and wild-type lysozymes.** (A) In the main chain: wild type (thin line), D52A (thick line); (B) in the region of the active site cleft: wild type (thin line), D52A (thick line).

alkaline limb had shifted to a lower pH, whereas the activity of D52A above pH 6.0 could not be determined because of the anomalous reaction. Inoue *et al.* have reported that the pK_a of Glu 35 in D52N is 5.0 (*28*). We determined the pK_a values of Glu 35 in D52A and the wild-type by tryptophyl fluorescence titration according to the method of Inoue *et al.* (*28*), they being 4.7 and 6.3, respectively. Thus, the alkaline limb of the pH-activity profile of D52A also reflects the pK_a of Glu 35.

X-Ray Structure of D52A—The X-ray crystallographic structure of D52A is shown in Fig. 5. For comparison, that of the wild-type is overlaid. Although a shift of the main-chain of D52A was observed at residue 47, the structure of D52A is essentially identical to that of the wild-type (Fig. 5A). This was also observed for D52S, as reported by Hadfield *et al.* (*14*). As shown in Fig. 5B, the structure around the mutated site in D52A is also almost identical to that in the wild-type. These results strongly indicated that the drastic decrease in activity was not due to the structural

change induced by the mutation but the mutation of Asp 52 itself.

DISCUSSION

We constructed a system for the expression and secretion of mature chicken lysozyme by *S. cerevisiae* using pAM82 as an expression vector and the yeast invertase signal peptide as a signal for secretion. The lysozyme gene was fused to the promoter of the acid phosphatase (*PHO5*) gene, which can be controlled through depletion of phosphate in the medium. The signal peptide of yeast invertase was also introduced for secretion of lysozyme expressed in yeast cells. The amount of lysozyme secreted into the medium was 4 mg/liter after cultivation at 30°C for 125 h (Fig. 2), and the purified lysozyme was found to be identical to native chicken lysozyme (*28*). Therefore, we concluded that *S. cerevisiae* can correctly process and effectively secrete a chicken lysozyme hybrid protein containing the yeast invertase signal peptide.

In the course of this study, we constructed an intermediate secretion-signal cassette vector, pKP1700, which contains the DNA sequence corresponding to *Sal*I-(invertase signal sequence)-*Eco*RI-*Sac*I-*Kpn*I-*Sma*I-*Bam*HI-*Xba*I-*Xho*I-*Pst*I-*Sph*I-*Hin*dIII-*Sal*I. The main advantages of pKP1700 are as follows. (i) Since pKP1700 contains useful multicloning sites, various DNA fragments encoding foreign proteins could be easily cloned at the multicloning sites of the vector. (ii) Based on the M13 phage vector, adjustment of the frame to obtain the fused gene between the yeast invertase signal peptide DNA and the mature region of the desired cDNA in the vector by single site-directed mutagenesis and DNA sequencing by the dideoxy method can easily be performed. (iii) The *Sal*I fragment, which contains the fused gene, can be cloned directly at the *Xho*I site of pAM82 for processing and secretion of the gene product as a mature form by yeast or at the *Sal*I site of another yeast-*E. coli* shuttle vector, pYG100, carrying the GPD (glyceraldehyde 3′-phosphate dehydrogenase) promoter (*7, 8*), for overproduction of the gene product (*30*). This secretion-signal cassette vector/pAM82 system was also successfully applied to the production of the extracellular domain of human tissue factor (TF) (*31*) and rat lysosomal acid phosphatase (Fujita, H., unpublished observation) in *S. cerevisiae*.

Since our secretion-signal cassette vector provides a simple and quick means for the expression and secretion of foreign proteins by yeast, it would be generally useful in situations in which it is desirable to produce foreign proteins with the native NH_2-terminus and the correct tertiary structure in yeast, and to produce foreign proteins that are toxic to yeast or unstable when produced through direct expression in the cytoplasm.

As can clearly be seen in Table II on the mutation of Asp 52 to Ala, Thr, Cys, or Ser, the activity decreased drastically (0.2-1.5%), but was evidently retained to some extent. The result means that Asp 52 does not play a critical role in lysozyme catalysis, such as a nucleophile to form a covalent intermediate, but that it definitely assists the catalytic reaction. This was consistent with the previous results (*10-12*).

On the hydrolysis of $(NAG)_6$ by the wild type, a bell-shaped pH-activity profile with an optimum at pH 5.0 was seen (Fig. 4). Since the pK_a values of Asp 52 and Glu 35 are 3.5 and 6.1, respectively (*9*), the acidic and alkaline limbs of the pH-activity profile may depend on Asp 52 and Glu 35, respectively. On the other hand, in the case of D52A, the pH-dependence of the activity in the acidic region disappeared. This may be interpreted by the disappearance of the participation of the dissociated form of Asp 52 on the mutation of Asp to Ala. Moreover, from the shift of the alkaline limb of the pH-activity profile to a lower pH, it was suggested that the dissociation of Glu 35 had been affected by Asp 52. When we measured pK_a of Glu 35 in D52A in the presence of $(NAG)_3$, the value was 4.7, which is close to the intrinsic pK_a of glutamic acid, 4.5. This result indicated that the alkaline limb of the pH-activity profile of D52A was due to a decrease in pK_a of Glu 35, and that Glu 35 evoked low but distinct catalytic activity in D52A. Moreover, this also supported that an electrostatic factor derived from Asp 52 was suggested to increase the pK_a of Glu 35 through the hydrogen-bonds of a water molecule, which is consistent with our previous paper (*28*). From the above results, it was confirmed, as originally suggested (*10*) for the catalytic mechanism, that Asp 52 assists catalysis by producing an electrostatic field in a dissociated form and by stabilizing the oxocarbonium ion intermediate.

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