Isolation and Characterization of 101-Succinimide Lysozyme That Possesses the Cyclic Imide at Asp101-Gly102

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ABSTRACT: Lytic activity of lysozyme solution gradually increased on incubation at pH 4, 40 °C. When the solution was analyzed by use of cation-exchange HPLC at pH 5, a new peak appeared with increased incubation time. The derivative in the new peak was identified to be 101-succinimide lysozyme in which cyclic imide formed at Asp101-Gly102. The formation of 101-succinimide lysozyme increased with increases in concentration of acetate buffer. Kinetic analysis of the formation of 101-succinimide lysozyme indicated that the cyclic imide was stable below pH 5 due to suppression of the hydrolysis of cyclic imide. Its lytic activity against *M. luteus*, which has a negative charge, was 165% at pH 7, whereas its activity against glycol chitin, which has no charge, was 90%. Since the lytic activity of Asn101 lysozyme, where one negative charge is eliminated, reached a maximum of 125%, it was suggested that the increase of lytic activity against bacterial cells in 101-succinimide lysozyme was due not only to the disappearance of the negative charge at Asp101 but also to the removal of steric hindrance at the upper part of the active site cleft.

Modern genetic engineering techniques and/or chemical synthesis allow us to synthesize polypeptides at will. However, as has already been reported (Harding, 1985), nonenzymatic reactions sometimes take place at side chains of amino acids, and enzymatic function may be influenced by these undesirable chemical reactions. Moreover, for *de novo* design of proteins, it is useful to obtain information on nonenzymatic reactions. To date, deamidations of asparagine and glutamine have been most extensively studied among the chemical reactions in proteins and peptides (Thannhauser & Scheraga, 1985; Tyler-Cross & Schirch, 1991; Wright, 1991). Namely, the deamidation *via* cyclic imide which is derived from the Asn-Gly sequence is well-known. On the other hand, the cyclic imide, which is derived from Asp-Gly, was observed in a peptide (Ondetti et al., 1968) and a protein (Murray & Clarke, 1984; Teshima et al., 1991). However, there is little information on its formation and stability because sufficient amounts of peptides and/or proteins possessing cyclic imide could not be obtained to characterize the product.

In this study, we have isolated large amounts of lysozyme derivative in which cyclic imide formed at Asp101 and Gly102 and have characterized the cyclic imide.

EXPERIMENTAL PROCEDURES

Materials. Hen egg white lysozyme was isolated from hen egg white and recrystallized five times. *Micrococcus luteus* was obtained from Sigma. Glycol chitin was prepared as described elsewhere (Yamada & Imoto, 1981). Chitin-coated Celite, an affinity adsorbent for lysozyme derivatives, was prepared as described elsewhere (Yamada et al., 1985a). A trimer of *N*-acetyl-D-glucosamine, $(NAG)_3$,[1] was prepared according to the method of Rupley (1964). Sephadex G-25 and CM-Toyopearl 650S were the products of Pharmacia and Tosoh (Tokyo, Japan), respectively. A column of Wakosil 5C18-200 (4.6 × 250 mm) was from Wako Pure Chemicals Institute (Osaka, Japan). All other reagents were of the highest purity commercially available.

Analytical Methods. Lytic activities of lysozyme derivatives against *M. luteus* were determined turbidimetrically at 450 nm in 0.05 M potassium phosphate buffer at pH 7, 30 °C (Inoue et al., 1992). The activities of the lysozyme derivatives against glycol chitin were determined in 0.1 M acetate buffer at pH 5.5, 40 °C (Yamada & Imoto, 1981).

Ion-exchange HPLC for analysis of lysozymes was accomplished on a column of CM-Toyopearl 650S at pH 5. The column (4 × 500 mm) was eluted with 20 mL of 0.1 M acetate buffer and 20 mL of the same buffer containing 1.0 M NaCl, pH 5, at a flow rate of 0.5 mL/min.

N-Terminal sequences of peptides were determined with an Applied Biosystems Model 470A gas-phase protein sequencer. FAB mass spectra of peptides were measured with a JEOL JMS DX-300.

Digestion of reduced and S-carboxymethylated lysozymes with L-1-(*p*-tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin; Sigma) and separation of the resultant tryptic peptides by RP-HPLC on a column of Wakosil 5C18-200 (4.6 × 250 mm) were performed as described previously (Inoue et al., 1992).

Analytical affinity chromatography of lysozyme derivatives on a chitin-coated Celite column (4 × 50 mm) using HPLC

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[1] Abbreviations: $(NAG)_3$, β(1–4)-linked trimer of *N*-acetyl-D-glucosamine; RP-HPLC, reversed-phase high-performance liquid chromatography; 101-β lysozyme, a lysozyme derivative in which the α-aspartylglycyl sequence at Asp101-Gly102 was converted to a β-linkage; 101-succinimide lysozyme, a lysozyme derivative in which the aspartylglycyl sequence at Asp101-Gly102 was converted to cyclic imide; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

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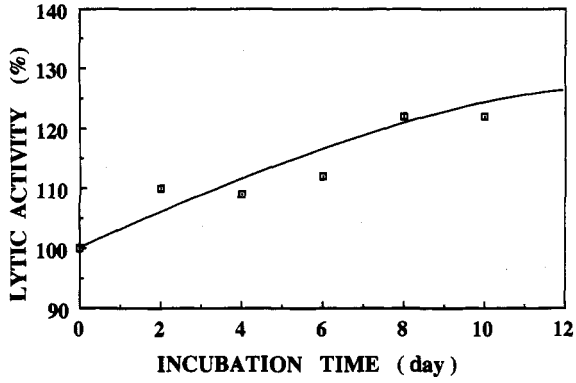


FIGURE 1: Time course of activation of lysozyme on incubation in 0.5 M acetate buffer at pH 4, 40 °C. Activity was determined against *M. luteus* at pH 7, 30 °C.

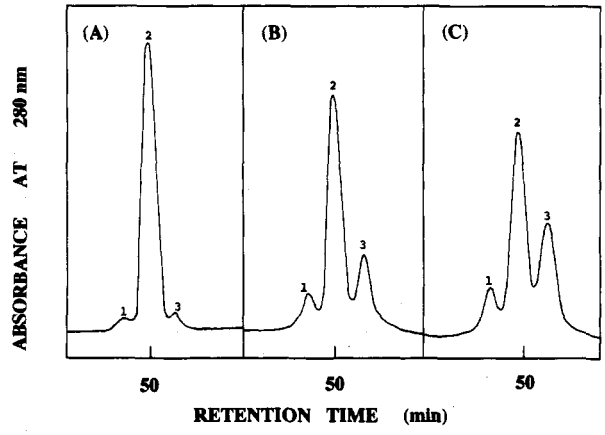


FIGURE 2: Ion-exchange HPLC of lysozyme incubated in 0.1 M acetate buffer at pH 4, 40 °C, on CM-Toyopearl 650S (4 × 500 mm). The column was eluted with 20 mL of 0.1 M acetate buffer and 20 mL of the same buffer containing 1.0 M NaCl at pH 5 and a flow rate of 0.5 mL/min. Lysozyme was incubated for 3 h (A), 6 days (B), and 10 days (C).

was performed at 0 °C as described previously (Yamada et al., 1985a).

The dissociation constants of lysozymes for binding to $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5, 40 °C, were determined by UV difference spectroscopy with a Hitachi 150-20 double-beam spectrophotometer (Inoue et al., 1992).

Preparation of Asn101 Lysozyme. Asn101 lysozyme was prepared according to the literature (Yamada et al., 1981) by the reaction of lysozyme with $(NH_4)_2SO_4$ at pH 5 in the presence of EDC.

RESULTS

Activation of Lytic Activity of Lysozyme by Incubation under Mildly Acidic Conditions. When lysozyme dissolved in 0.5 M acetate buffer at pH 4 (0.5 mg/mL) was incubated at 40 °C, the lytic activity against *M. luteus* gradually increased and reached 125% relative to the initial activity after incubation for 10 days at pH 4 (Figure 1).

Structural Analysis of Lysozyme Incubated at pH 4 and 40 °C. In order to elucidate the reason for the increase in lytic activity of lysozyme, aliquots of lysozyme solution following appropriate incubation times were analyzed using cation-exchange HPLC (Figure 2). Peak 3 in Figure 2, which was eluted later than native lysozyme (peak 2), increased with incubation at pH 4, 40 °C. The lysozyme derivatives in peak 1 were shown to be 101-β lysozyme, that is, a lysozyme derivative in which the α-aspartylglycyl bond at 101–102 is converted to a β-linkage, when compared with the authentic sample (Yamada et al., 1985b). The lytic activities of 101-β lysozyme and the lysozyme derivative in peak 3 were 120% and 165%, respectively, relative to that of peak 2 (native lysozyme) (Table 1). Thus, the increase in lytic activity with incubation time at pH 4, 40 °C, was shown to be mainly due to production of the derivative in peak 3. On the other hand, Asn101 lysozyme, where the negative charge at Asp101 was eliminated and which was eluted later than native lysozyme on ion-exchange HPLC at pH 5, showed lytic activity of 123% relative to that of native lysozyme (Table 1).

Table 1: Lytic Activity of Lysozymes Produced under Mildly Acidic Conditions

lysozyme	activity (%)	lysozyme	activity (%)
peak 1 (101-β)	120 ± 3	peak 3	165 ± 5
peak 2 (native)	100	101 Asn	123 ± 2

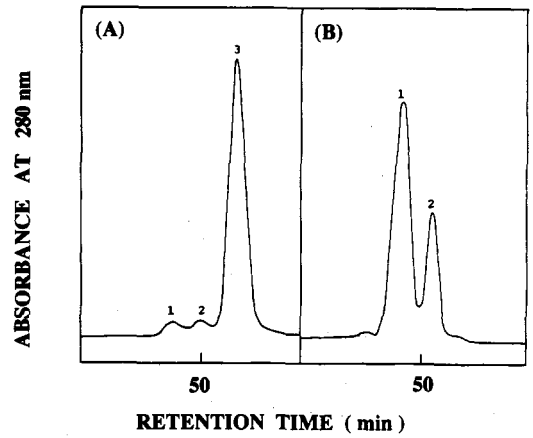


FIGURE 3: Ion-exchange HPLC of the lysozyme derivative on CM-Toyopearl 650S (4 × 500 mm). Before (A) and after (B) incubation of the lysozyme derivative in peak 3 of Figure 2 in 0.1 M phosphate buffer at pH 8, 40 °C, for 5 h. The column was eluted with 20 mL of 0.1 M acetate buffer and 20 mL of the same buffer containing 1.0 M NaCl, pH 5, at a flow rate of 0.5 mL/min.

The derivative in peak 3 was incubated in 0.1 M phosphate buffer at pH 8, 40 °C for 5 h and was subjected to cation-exchange HPLC. As shown in Figure 3, the derivative in peak 3 was completely converted to 101-β and native lysozyme (proportion, 2:1) by incubation at mildly basic conditions. The derivative in peak 3 was reduced by 2-mercaptoethanol, carboxymethylated, and digested in 0.1 M phosphate buffer at pH 8.0 by TPCK-trypsin according to the literature (Okazaki et al., 1982). The resultant tryptic peptides were analyzed on reversed-phase HPLC (Figure 4A). For comparison, the pattern derived from native lysozyme is also shown in Figure 4B. The pattern of the tryptic peptides resulting from the derivative in peak 3 was identical with that resulting from native lysozyme with the exception of the peptide eluted just before the T_{13} (Ile98–Arg112) peptide, while the height of the T_{13} peptide was reduced. On the basis of earlier reports (Yamada et al., 1985b), the new peak eluted just before T_{13} peptide was identified to be a peptide with β-linkage at Asp101-Gly102 (T_{13}-β peptide). From these results (Figures 3 and 4), it was suggested that the derivative in peak 3 in Figure 2 was unstable under basic conditions, but stable under mildly acidic conditions, an intermediate in the formation of 101-β lysozyme.

Identification of Succinimide Derivative in Tryptic Peptide Containing Aspartylglycyl Sequence. It was difficult to directly determine whether the derivative in peak 3 in Figure 2 was an intermediate in the formation of 101-β lysozyme.

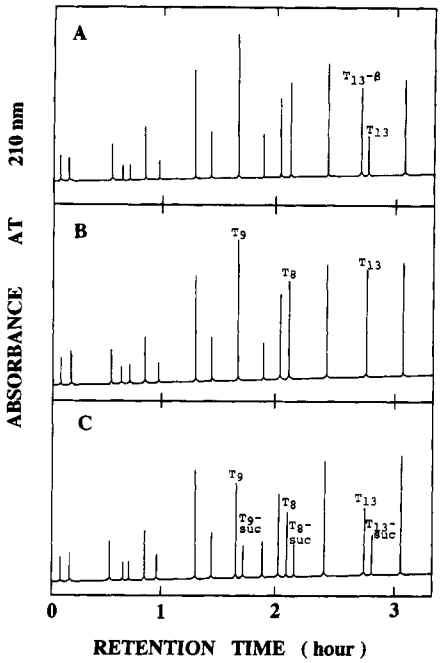


FIGURE 4: Reversed-phase HPLC of tryptic peptides derived from reduced and carboxymethylated lysozymes on Wakosil 5C18-200 (4.6 × 250 mm). The column was eluted with a gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 1.2×10^{-2} N concentrated HCl at a flow rate of 0.4 mL/min. (A) 101-succinimide lysozyme; (B) native lysozyme; (C) tryptic peptides incubated in 0.1 M acetate buffer at pH 4, 40 °C for 70 h. T_9-suc, T_8-suc, and T_{13}-suc indicate succinimide of T_9, T_8, and T_{13}, respectively.

Thus, tryptic peptides derived from native lysozyme were incubated in 0.1 M acetate buffer at pH 4, 40 °C, for 70 h to examine the formation of the intermediate (succinimide derivative) in the peptide containing the aspartylglycyl sequence. The elution patterns of tryptic peptides after incubation at pH 4, 40 °C, for 70 h are shown in Figure 4C. Three new peaks eluted just after peaks T_8 (Asn46–Arg61), T_9 (Trp62–Arg68), and T_{13} which possess the Asp-Gly sequence appeared, while the amounts in peaks T_8, T_9, and T_{13} decreased. Because there are three aspartylglycyl sequences (Asp48-Gly49, Asp66-Gly67, and Asp101-Gly102) in lysozyme, it was suggested that the peptides containing the sequence are easily converted into succinimide derivatives at acidic conditions. From amino acid analyses of these peptides, the peaks were found to have been derived from T_9, T_8, and T_{13}, respectively. Mass spectra indicated that the molecular masses of these peaks were smaller by 18 daltons than those of intact T_9, T_8, and T_{13}, respectively. Following incubation of these peptides under mildly basic conditions, both peaks after T_9 and T_{13} gave two peaks which were identified respectively as α and β peptides according to the literature (Yamada et al., 1985b), but the peak after T_8 gave only one peak on RP-HPLC under the routine conditions described above. On the other hand, when incubated under mildly basic conditions and eluted with 1.0×10^{-3} N HCl instead of 1.2×10^{-2} N HCl used under routine conditions, the peak after T_8 separated into two peaks, as was observed in the peaks after T_9 and T_{13}. N-Terminal analyses of these two separated peptides were carried out by use of a gas-phase peptide sequencer. As shown in Figure 5, the aspartylglycyl sequence could be detected in one peptide but not in the other. Thus, the peptide after T_8 was also converted into intact T_8 and T_8-β under alkaline conditions, as observed in the peptides after T_9 and T_{13}. Therefore, the molecular mass (−18 daltons) and conversion into α and β Asp-Gly under alkaline conditions

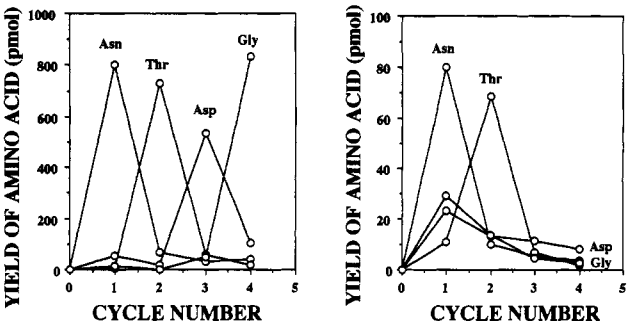


FIGURE 5: N-Terminal analyses of T_8 and T_8-β peptides by gas-phase protein sequencer. These peptides were obtained by an RP-HPLC system where the eluting solvent contained 1.0×10^{-3} N HCl after incubation of T_8 succinimide under alkaline pH.

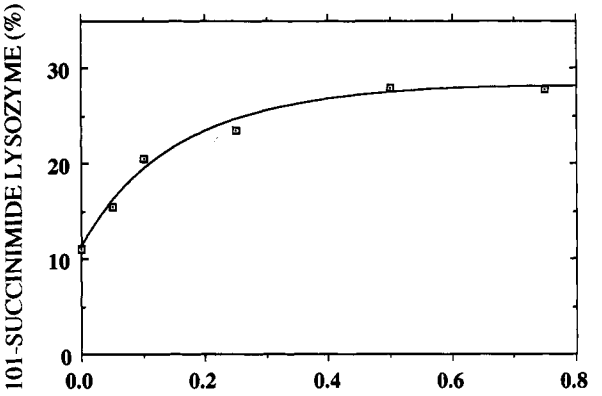
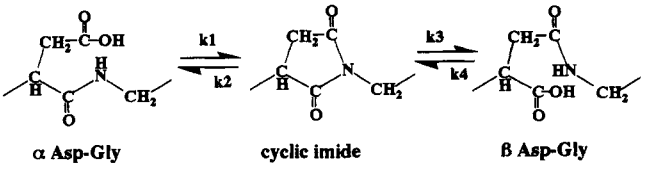


FIGURE 6: Yield of 101-succinimide lysozyme after incubation at pH 4.0, 40 °C, for 6 days at various concentrations of acetate buffer.

Scheme 1: Isomerization of Asp-Gly Sequence



indicated that all three aspartylglycyl sequences in tryptic peptides of lysozyme were found to form cyclic imide after incubation in acetate buffer at pH 4, 40 °C. Thus, it was concluded that the derivative in peak 3 in Figure 2 was a lysozyme derivative which possessed cyclic imide at Asp101-Gly102 (101-succinimide lysozyme).

Effect of Acetate Buffer on the Formation of 101-Succinimide Lysozyme. To examine the effect of acetate buffer on the formation of 101-succinimide lysozyme, native lysozyme was incubated in various concentrations of acetate buffer at pH 4, 40 °C, for 6 days. The amounts of 101-succinimide lysozyme were determined by cation-exchange HPLC. As shown in Figure 6, the formation of 101-succinimide lysozyme increased with the concentration of acetate buffer.

pH Dependence of the Isomerization Kinetics at Asp101-Gly102 in Lysozyme. Kinetic parameters of conversion among three species in Scheme 1 can be presented as:

$$v_{\alpha 0} = k_1[\alpha]_0 \quad (1)$$

$$v_{\beta 0} = k_4[\beta]_0 \quad (2)$$

$$v_{suc0} = (k_2 + k_3)[suc]_0 \quad (3)$$

$$k_2 = k_1[\alpha]_{equi}/[suc]_{equi} \quad (4)$$

$$k_3 = k_4[\beta]_{equi}/[suc]_{equi} \quad (5)$$

where $v_{\alpha 0}$, $v_{\beta 0}$, and v_{suc0} are the initial decreasing velocity of α and β Asp-Gly and succinimide (cyclic imide), respectively; $[\alpha]_{equi}$, $[\beta]_{equi}$, and $[suc]_{equi}$ are equilibrium values of α and β Asp-Gly and succinimide, respectively. Thus, distributions of native, 101-β, and 101-succinimide lysozyme after incubation of native lysozyme in 0.2 M acetate buffer (0.5 mg/mL) at 40 °C using various pH values (pH 3–5.5), for 2800 h (about 4 months) where they are at equilibrium, were determined by use of cation-exchange HPLC (no visible precipitate after 4 months). The percentages of these species at equilibrium in various pHs are shown in Table 2. On the other hand, native, 101-β and 101-succinimide lysozyme were incubated in 0.2 M acetate buffer (0.5 mg/mL) at pH 3–5.5, and the initial rate constant of the decrease of each lysozyme at various pHs was evaluated by analyzing aliquots of each solution at appropriate time intervals on the cation-exchange HPLC described above. In the acidic pH range, the rate constants of isomerization at Asp101-Gly102 corresponding to Scheme 1 were evaluated by eqs 1–5 and are shown in Figure 7. This result indicated that the stability of the succinimide intermediate at pH 4 was due to suppression of the hydrolyzing rate constant into α- and β-aspartylglycine (k_2 and k_3).

Activity and Affinity of 101-Succinimide Lysozyme against Simple Substrates. Glycol chitin is a simple substrate for lysozyme. The enzymatic activity of 101-succinimide lysozyme against glycol chitin at pH 5.5, 40 °C, was measured (Table 3). The activity of 101-succinimide lysozyme against glycol chitin was slightly lower than that of native lysozyme. These results suggested that the modification of Asp101, which is a substrate binding residue (Imoto et al., 1972), affected the binding ability to chitin. On the other hand, the affinity of 101-succinimide lysozyme against chitin was investigated by affinity-HPLC equipped with a column of chitin-coated Celite. The elution pattern of 101-succinimide lysozyme is shown in Figure 8B. For comparison, those of native, 101-β, and the mixture of three lysozymes are shown in Figure 8A, -C, and -D, respectively. These results indicated that the decrease in the activity of 101-succinimide lysozyme against glycol chitin was proportional to the reduction in binding ability. Moreover, the dissociation constant of 101-succinimide lysozyme for binding to $(NAG)_3$ at pH 5.5, 40 °C, was investigated (Table 3). The binding ability of lysozyme to $(NAG)_3$, which is known to interact with Asp101 (Blake et al., 1967), also decreased by forming cyclic imide at positions 101 and 102.

DISCUSSION

In the reaction of lysozyme with EDC and imidazole, Yamada et al. (1985b) suggested that the β-aspartylglycyl sequence at Asp101-Gly102 may form *via* cyclic imide. However, cyclic imide at Asp101-Gly102 was not detected. In that report, lysozyme was reacted with EDC and imidazole at pH 5 and was dialyzed against 0.02 M borate buffer at pH 10 in order to hydrolyze acylimidazole. Thus, the authors detected no 101-succinimide lysozyme because the hydrolysis of the cyclic imide increased with increases in pH (Figure 7). As is shown in Figure 7, the stability of 101-succinimide lysozyme under mildly acidic conditions was evoked by a small rate constant for hydrolysis of the cyclic imide. Moreover,

Table 2: Equilibrium Values of Isomerization of Asp101-Gly102 in Acidic pH Region at 40 °C

pH	equilibrium values (%)		
	native	101-succinimide	101-β
3.0	37.9 ± 0.1	49.2 ± 0.4	12.9 ± 0.3
3.5	38.6 ± 1.9	42.4 ± 0.1	19.0 ± 1.8
4.0	41.3 ± 1.4	30.3 ± 1.1	28.4 ± 1.0
4.5	41.8 ± 2.5	22.3 ± 3.1	35.9 ± 4.2
5.0	46.3 ± 2.3	16.0 ± 1.7	37.7 ± 2.1
5.5	49.2 ± 0.2	9.4 ± 1.2	41.4 ± 0.2

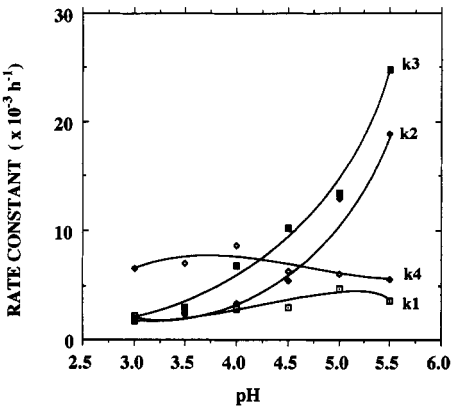


FIGURE 7: pH dependence of rate constants in conversion among the three species (native, 101-succinimide, and 101-β lysozyme; see Scheme 1).

Table 3: Activities against Glycol Chitin and Affinities for $(NAG)_3$ of Native and 101-Modified Lysozyme

lysozyme	GC (%)[a]	K_d for $(NAG)_3$ ($\times 10^{-5}$ M)[b]
native	100	1.4 ± 0.1
101-β	97 ± 1[c]	8.5 ± 0.1
101-succinimide	89 ± 2	6.4 ± 0.3

[a] Activity against glycol chitin in 0.1 M acetate buffer at pH 5.5, 40 °C. [b] Dissociation constant of the complex between lysozyme and $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5, 40 °C. [c] Reported previously (Ueda et al., 1987).

as the cyclic imide was stable under the separating conditions (pH 5), we could obtain a lysozyme derivative in which cyclic imide formed at Asp101-Gly102 (101-succinimide lysozyme) (Figure 2). The formation of 101-succinimide lysozyme increased with increases in concentration of acetate buffer (Figure 6). Thus, we should be cautious when treating lysozyme in the presence of high concentrations of acetic acid.

Lysozyme has three Asp-Gly sequences at 48–49, 66–67, and 101–102. In the folded state of lysozyme, cyclic imide only formed at Asp101-Gly102, but in the tryptic peptides derived from lysozyme, cyclic imides formed at all three Asp-Gly sequences. The difference in the magnitude of formation of cyclic imide in the folded state of lysozyme is likely due to the difference in accessibility of the side chain at Asp-Gly to solvent (Blake et al., 1967). These observations were consistent with the fact that the Asp-Gly sequence at 101–102 in the folded state of lysozyme is located in a flexible loop and is highly accessible to solvent (Imoto et al., 1972). Therefore, the environment around Asp101-Gly102 in the folded state of lysozyme should be similar to that in the denatured state because all Asp-Gly sequences in the tryptic peptides were converted to the cyclic imide under mildly acidic conditions. These results strongly indicate that in general the cyclic imide formed easily at the Asp-Gly sequence under mildly acidic conditions when the sequence was highly accessible to solvent. On the other hand, Wearne and Creighton (1989) reported

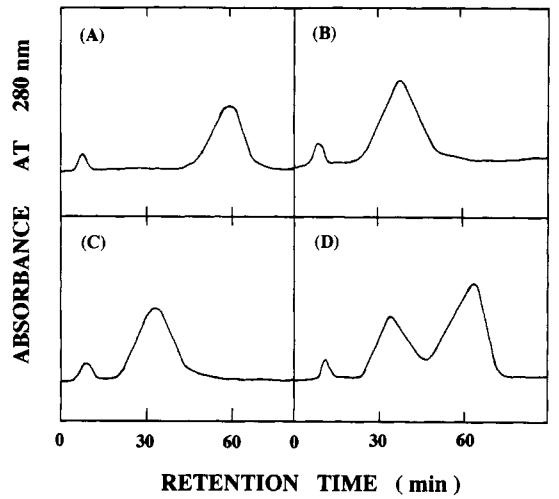


FIGURE 8: Affinity-HPLC of lysozymes at 4 °C. The column was eluted with a gradient formed of 20 mL of 0.1 M acetate buffer at pH 5.5 containing 0.25 M NaCl and 20 mL of 1.0 M acetic acid containing 0.25M NaCl. (A) Native; (B) 101-succinimide; (C) 101-β lysozyme; (D) mixture of native, 101-β, and 101-succinimide lysozyme in Figure 2B.

an effect of a local conformation on the rate of deamidation at Asn67-Gly68 in ribonuclease A. Even though the labile Asn67 residue was located on the surface of ribonuclease A, the rate of deamidation of Asn67 in folded ribonuclease A was one-thirtieth than that of unfolded ribonuclease A at 37 °C, pH 8. This result was interpreted by restriction of the flexibility of the main chain around Asn67-Gly68 evoked by the secondary structure and by a disulfide bond (Cys65–Cys72). Consequently, these results indicated that the formation of the cyclic imide may require not only the accessibility of a side chain to solvent but also flexibility of the main chain. When we measured the rate of formation of the cyclic imide at pH 4 in tryptic peptide T_{13} which contains Asp101-Gly102 [$k(T_{13}) = 6.3 \times 10^{-3}$ h^{-1}], the maximum rate was twice that in lysozyme [$k(N) = 2.8 \times 10^{-3}$ h^{-1}]. This result indicated that the loop region around Asp101-Gly102 not only is accessible to the solvent but also is sufficiently flexible. Thus, these findings of intraconversion at Asp101-Gly102 may be applicable to other proteins which possess this sequence.

Typical X-ray crystallography of lysozyme was performed using lysozyme crystal obtained in 5 mM acetate buffer at pH 4.5, 4 °C, containing 0.5% NaCl (Blake et al., 1967). The *B* factor around the loop region containing Asp101-Gly102 was reported to be abnormally larger than the average value in lysozyme molecule (Diamond, 1974). The amount of 101-β and 101-succinimide lysozymes in lysozyme crystal that we prepared under the above conditions for about 1 month was approximately 35%. Although subtle differences in preparation of the crystal may have had some effects, the reason for the increase in the *B* factor around the loop region containing Asp101-Gly102 may be explained by an intraconversion among native, 101-succinimide, and 101-β lysozyme.

The binding ability of 101-succinimide lysozyme with $(NAG)_3$ and chitin-coated Celite was smaller than that of native lysozyme. This suggested that hydrogen bonding between the lysozyme molecule and substrate analogues in 101-succinimide lysozyme decreased because the carboxylic acid of Asp101 in lysozyme binds with the substrate analogue in hydrogen bonds, as revealed by X-ray crystallographic data (Imoto et al., 1972). The decrease in activity against glycol chitin may also be due to the same reason. On the other hand, the activity against *M. luteus* increased more than that of native lysozyme. The increase in activity may have resulted from a decrease of unfavorable electrostatic interaction due to the disappearance of negative charges at Asp101, which diminishes electrostatic repulsion between carboxylic acid at Asp101 on the surface of lysozyme and negative charge on the cell wall of *M. luteus*. However, since the elimination of one negative charge increased lytic activity a maximum of 20%, as seen in Asn101 lysozyme, there is likely another reason for the increase in lytic activity of 101-succinimide lysozyme. To date, similar phenomena, that is, increasing activity against *M. luteus* and decreasing activity against glycol chitin, were observed in mutant lysozymes where Asp101 was replaced by Ser or Ala (Imoto et al., 1989) and where Trp62 was replaced by Tyr (Kumagai et al., 1987). Common characterizations of these mutations are that the residues are located in the upper part of the active site cleft of lysozyme (Blake et al., 1967) and the size of the side chain is reduced. Therefore, the removal of steric hindrance on the side chain of the residues of the upper part of the active site cleft would contribute to increased lytic activity of lysozyme.

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