

The Amino Acid Sequence of Reeves' Pheasant (*Syrmaticus reevesii*) Lysozyme

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Received November 13, 1990

The amino acid sequence of reeves' pheasant lysozyme was analyzed. Carboxymethylated lysozyme was digested with trypsin and resulting peptides were analyzed using the DABITC/PITC double coupling manual Edman method. The established amino acid sequence had seven substitutions, Tyr3, Leu15, His41, His77, Ser79, Arg102, and Asn121, compared with hen egg-white lysozyme. Ser79 was the first found in a bird lysozyme. A substitution in the active site was found in position 102 which has been considered to participate in the substrate binding at subsites A–C.

Lysozyme is one of the most characterized hydrolases, which cleave β -1,4 linkage of *N*-acetylglucosamine (GlcNAc) homopolymer or GlcNAc-*N*-acetylmuramic acid heteropolymer. This enzyme is composed of 129–130 amino acids, and the tertiary structure of hen egg-white lysozyme (HEWL) has been elucidated by X-ray refraction study.¹⁾ The mechanism of catalytic reaction of this protein has also been elucidated in detail. The enzyme contains six substrate binding subsites (A–F) that can bind each saccharide unit. Substrate molecules are catalytically hydrolyzed at subsites D and E. On the other hand, for the transglycosylation activity of lysozyme, two substrate binding modes were proposed at subsite E–F as “left side” and “right side” by energy minimization^{2,3)} or an immunological method.⁴⁾ However, the amino acid residue which participates in the substrate binding at subsite E–F was not identified. Recently, we reported the enhancement of the transglycosylation activity by the chemical modification of Asp101 and Trp62.⁵⁾ In this modification, we found that the binding free energy was decreased at subsite A in the modified lysozyme

and concluded that the enhancement of the transglycosylation was correlated with the binding of substrate at subsite A. The substrate binding at subsite A has further been investigated by an NMR study.⁶⁾ The covalently bound glucosamine on Asp101 at subsite A caused a change of the indole proton of Trp62. This result strongly suggested that the amino acid at position 101 participates in the interaction between the substrate and substrate binding site of lysozyme. Therefore, a lysozyme carrying a substituted amino acid at the active site or a amino acid susceptible to chemical modification at the active site is considered to be available for investigations of the participation of amino acids in the transglycosylation activity.

In this paper, we report the amino acid sequence of lysozyme from reeves' pheasant (*Syrmaticus reevesii*). By comparing the amino acid sequence of reeves' pheasant lysozyme (RPL) and other pheasants, we found the amino acid substitution at subsite A (position 102) which will participate to the enzyme action of lysozyme.

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Abbreviations: DABITC, 4-*N,N*-dimethylaminoazobenzene 4'-isothiocyanate; PITC, phenylisothiocyanate; TPCK, tosylphenylalanylchloromethyl ketone; RPL, reeves' pheasant lysozyme; IPL, Indian peafowl lysozyme.

Materials and Methods

Eggs. Freshly laid reeves' pheasant, Lady Amherst's pheasant, golden pheasant, Indian peafowl, Japanese pheasant, and guineafowl eggs were obtained from The Kumamoto Zoological Park, Kumamoto, Japan. Hen egg-white lysozyme was purchased from Seikagaku Kogyo Co., Japan.

Purification of lysozyme. A water extract of egg white was treated with isoelectric precipitations at pHs 4.0 and 7.0 and the clarified solution was then put on a CM-Toyopearl column (1.5 × 46 cm) equilibrated with 0.03 M phosphate buffer (pH 7.0). The column was developed with the stepwise elution of the same buffer containing 0.3 M NaCl. The lysozyme fraction was then rechromatographed on the same column with a gradient of 0.1 M to 0.3 M NaCl in the same buffer.

Peptide separation. Reduced and carboxymethylated lysozyme (Cm-lysozyme) was prepared by the method of Crestfield *et al.*⁷⁾ Cm-lysozyme was digested with TPCK–Trypsin (1/50, w/w, TR-TPCK, Cooper Bio-medical Co.) at pH 8.0 and 37°C for 4 hr. The tryptic digest was put on a reverse-phase (RP) HPLC column (C18 120A S-5, 4.0 × 250 mm, Yamamura Chemical Co., Japan) using a JASCO 800 series HPLC (Japan Spectroscopic Co., Japan). The details of the conditions for peptide separation were described previously.⁸⁾ Briefly, the peptides were developed with a gradient elution system of 0.1% TFA (solv. A) and 60% acetonitrile in 0.1% TFA (solv. B). A gradient of 0% to 50% of solv. B was used for 130 min after 10 min elution with solv. A.

Amino acid analysis and amino acid sequencing. Tryptic peptides were hydrolyzed in evacuated sealed tubes at

110°C for 20 hr with constant boiling HCl containing 0.05% β -mercaptoethanol. Resulting hydrolysates were analyzed with an amino acid analyzer (Model 835, Hitachi Co., Japan). The amino acids of tryptic peptides were sequenced using a DABITC/PITC double coupling manual micro sequencing method.^{9,10)}

Lysozyme activity. The activity was assayed using the lyophilized cell wall of *M. lyodeikticus* as a substrate. Namely, samples (10 to 100 μ l) of the eluent were added to 3 ml of the substrate suspension composed of substrate and 0.1 M phosphate buffer, pH 7.0 adjusted to OD 1.0 at 540 nm. One enzyme unit was defined as the amount causing a decrease of 0.1 absorbance unit at 540 nm which in the reaction for 1 min at 25°C.

Results

Purification of RPL

The elution profile of the final step of purification of RPL on CM-Toyopearl column is shown in Fig. 1. As the lysozyme was eluted in a peak with a shoulder, each fraction (A and B) was used for the subsequent investigation. Since both lysozyme fractions showed a single band on electrophoresis, they were lyophilized and used for structure analysis.

Amino acid sequence of RPL and comparison with other phasianid bird lysozymes

In the elution profile of HPLC of fraction B and other phasianid bird lysozymes (Lady Amherst's pheasant lysozyme, Indian peafowl

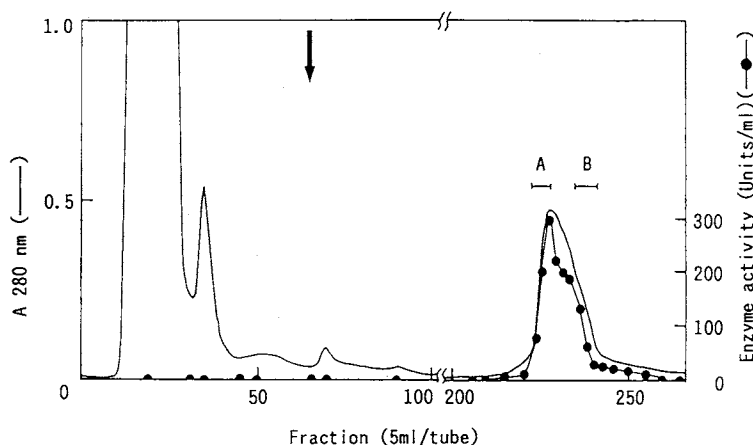


Fig. 1. Ion-exchange Chromatography of Reeves' Pheasant Lysozyme on CM-Toyopearl Column.

The elution was done with a linear gradient between 0.1 and 0.3 M NaCl in 0.03 M phosphate buffer (pH 7.0) from an arrow in the figure. Fractions A and B were structurally analyzed.

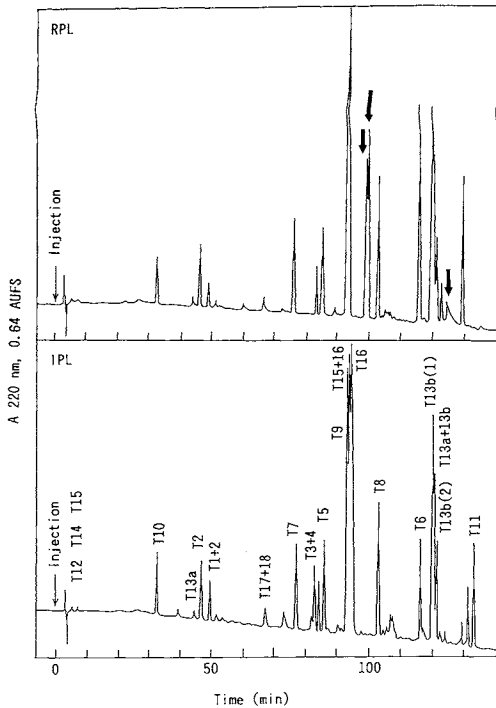


Fig. 2. Comparison of RP-HPLC Pattern of RPL with That of IPL.

The peptide in each peak was numbered by the method of Canfield.¹³⁾ Peaks in RPL indicated by arrow are the peaks appeared in different positions as compared with the IPL pattern. For detailed conditions of HPLC see the text.

lysozyme, Japanese pheasant lysozyme, guinea-fowl lysozyme, and HEWL), the highest homology was found between RPL and Indian peafowl lysozyme (IPL). Accordingly, the peptide maps of RPL and IPL were compared in Fig. 2. All peptides with the exception of T15+16, T16, and T11 were eluted in the same positions. This result indicates that the amino acid substitutions only occur in peptides T16 and T11. The completion of the sequencing was done by the following Edman degradation for all tryptic peptides. The amino acid sequence of fraction A was also analyzed, and proved to have the same amino acid sequence as fraction B with the exception of Asp103 instead of Asn103. In Fig. 3, the amino acid sequence of RPL is compared with reported amino acid sequences of other phasianid birds. Seven substitutions, Tyr3, Leu15, His41, His77, Ser79, Arg102, and Asn121 were found in RPL

as compared with HEWL. Four of 6 substitutions of the amino acid residues in RPL (Leu15, His41, Arg102, and Asn121) were exchanged by 2 point mutations. These variable positions evaluated from the variability of amino acid on lysozyme sequences were calculated by base change and amino acid exchange values. The results are shown in Table I. Base change values were represented by the combined values of the minimum base change number and classified into transversion and transition. No notable difference was observed between transversion and transition, but the total number of the base changes indicated flexible positions at 15, 41, 102, and 121. The amino acid exchange value was represented by the value of the distance matrix reported by Risler *et al.*²¹⁾ and the mutation data matrix by Schwartz *et al.*²²⁾ The increased number of these values implies that there is a high variability of amino acid exchange. The variable positions indicated by amino acid exchange values coincided with the results for the base change value except for the amino acids at positions 84 and 121, which were estimated by the mutation data matrix method.

Discussion

RPL was purified from eggs of reeves' pheasant and its amino acids were sequenced. In the purification step of RPL by ion-exchange chromatography on CM-Toyopearl, we found that rechromatography of fraction B also provided two fractions, A and B. The peptide maps of these two fractions showed a difference only in the elution positions of T13b. Namely, the peptide map of fraction B showed two peaks of T13b, one carries Asn103 (T13b(Asn)) and another carries Asp103 (T13b(Asp)), but the peptide map of fraction A showed one peak of T13b which carries Asp103 (Fig. 4). Based on this fact, it can be considered that fraction B was converted to fraction A during purification of RPL. Further, the one week stored egg white of RPL at 4°C had a single peak at the position of fraction A on

	-1	10	▼	20	30	40▼	50
RPL	--Y-----	-----L--	N--R-----	-----TH--	-----	-----	-----
IPL	--Y-----	-----L--	N--R-----	-----TH--	-----	-----	-----
LAPL, GPL	--Y-----	-----L--	N--R-----	-----TH--	-----	-----	-----
TEWL	--Y-----	-----L--	N--R-----	-----TH--	-----	-----	-----
HEWL	KVFGRC	ELAAAMKR	RHGLDNY	RGYSLGN	VWVCAAK	FESNFNT	QATNRNTDGS
JPL, RNPL	G--Y-----	-----M--	N--R-----	-----TG--	-----	-----	-----
JQL	--Y-----	-----H--	K--Q-----	-----TQ--	-----	-----	-----
BQL	--F-----	-----H--	N--R-----	-----SQ--	-----	-----	-----
CQL	--F-----	-----H--	N--R-----	-----SQ--	-----	-----	-----
GHL	--F-----	-----H--	N--R-----	-----SQ--	-----	-----	-----

	60	70	80	90	100
RPL	-----I-----	-----R--	-----R--H-S-----	-----L-----	-----SV-----V--
IPL	-----I-----	-----R--	-----R--N-P-----	-----L-----	-----SV-----V--
LAPL, GPL	-----I-----	-----R--	-----R--H-P-----	-----L-----	-----SV-----V--
TEWL	-----I-----	-----R--	-----K--N-P-----	-----L-----	-----SV-----A--
HEWL	TDYGIL	QINSRWWCNDG	RTPGSRNLCNIPCS	ALLSSDITASVNC	AKKIVS
JPL, RNPL	-----I-----	-----R--	-----K--H-P-----	-----L-----	-----SV-----V--
JQL	-----I-----	-----R--	-----R--N-P-----	-----L-----	-----SV-----V--
BQL	-----V-----	-----K--	-----R--N-P-----	-----L-----	-----TV-----V--
CQL	-----V-----	-----R--	-----R--N-P-----	-----L-----	-----TV-----V--
GHL	-----V-----	-----R--	-----R--N-P-----	-----Q-----	-----TA-----V--

	▼	110	120▼
RPL	DRN-----	NR-----	NA-IR-----
IPL	DRN-----	NR-----	HA-IR-----
LAPL, GPL	DGN-----	NR-----	NA-TR-----
TEWL	GGN-----	NR-----	HA-IR-----
HEWL	DNGMNAWVAWR	NRCKGTDVQ	AWIRGCRL
JPL, RNPL	DGN-----	KH-----	NV-IR-----
JQL	DVH-----	NR-----	NA-IR-----
BQL	DGN-----	NR-----	QA-IR-----
CQL	DGN-----	NR-----	HA-IR-----
GHL	DGN-----	KH-----	RV-IR-----

Fig. 3. Comparison of Amino Acid Sequences of Phasianid Bird Lysozymes.

Amino acid sequence of hen egg-white lysozyme and positions containing substituted amino acids are indicated by single letters. Other positions which contain no substituted amino acid are indicated by dashes. The abbreviations of lysozymes are as follows: reeves' pheasant lysozyme; RPL (this study), Indian peafowl lysozyme; IPL,⁸⁾ Lady Amherst's pheasant lysozyme; LAPL,¹¹⁾ golden pheasant lysozyme; GPL,¹¹⁾ turkey egg-white lysozyme; TEWL,¹²⁾ hen egg-white lysozyme; HEWL,^{13,14)} Japanese pheasant lysozyme; JPL,¹⁵⁾ ring-necked pheasant lysozyme; RNPL,¹⁶⁾ Japanese quail lysozyme; JQL,¹⁷⁾ bobwhite quail lysozyme; BQL,¹⁸⁾ California quail lysozyme; CQL,¹⁹⁾ guineafowl lysozyme; GHL.²⁰⁾ Hypervariable positions are indicated by arrows.

CM-Toyopearl chromatogram. These findings suggest that in the primary structure of RPL, the amino acid residue at position 103 is essentially Asn, and its side chain is readily deamidated during storage of eggs or purification of lysozyme, yielding fraction A. No significant difference of lysozyme activity was found in these two fractions when the cell walls were used as the substrate. Such a deamidation of the Asn residue has also been reported for Asn103 of Indian peafowl lysozyme.⁸⁾ The common sequence, Asp101-Arg102-Asn103, in RPL and peafowl lysozyme may be closely associated with the deamidation of the Asn residue of position 103.

By comparing the amino acid sequences of

lysozymes from phasianid birds, the substitution of Pro79 to Ser79 in RPL was first found in bird's lysozyme and only two vertebrate lysozymes, rat²³⁾ and rabbit²⁴⁾ lysozymes, were reported to contain Ser79. Arg102 was found only in RPL and peafowl lysozyme.

To calculate the variability of amino acids in each position, substituted amino acids were compared and evaluated by the combined number of facility of the amino acid exchange using the minimum base change value or the amino acid substitution data matrix (Table I). The positions 15, 41, 102, and 121 were shown to be variable for the amino acid substitution in these lysozymes, and position 84 was variable when counted by mutation data matrix. This

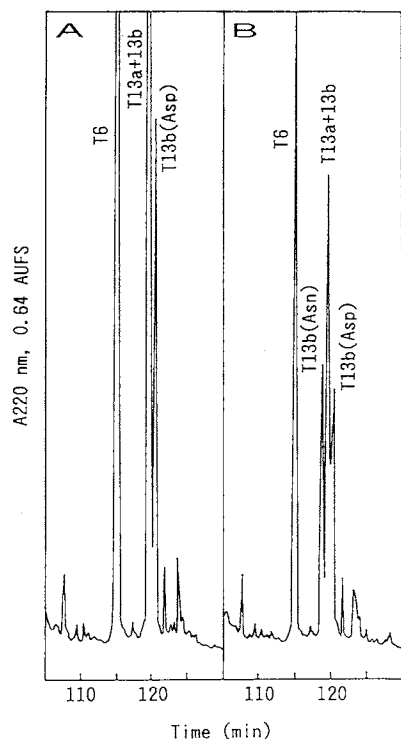


Fig. 4. The Elution Profiles of Peptide T13b from Fractions A and B.

The tryptic peptides from fraction A (A) and from fraction B (B) were separated by RP-HPLC with the same condition of Fig. 2 and elution positions of T13b were compared. The amino acids in the parenthesis indicate the amino acids at position 103 from the sequence analysis.

difference was assumed to be due to the difference of data estimation used. There is evidence that the known epitopes of human lysozyme²⁵⁻²⁸⁾ are contained in these hyper-variable positions except for the residue at position 121. As position 121 was assessed to be variable by two of three methods, this result indicates that the variable regions on the surface of the lysozyme molecule are recognized as epitopes. Such a relationship between the epitope and the variable region has been reported for cytochrome *c*.²⁹⁾

In these hyper-variable positions, only the amino acid residue at position 102 was at the substrate binding site. That is, Gly102 at the right-side of the subsite A-B of HEWL was replaced by Arg102 in RPL and peafowl lysozymes (Fig. 5).

X-ray crystallographic studies on HEWL have indicated that Asp101 and Gly102 are at subsite A. Fukamizo *et al.* reported that amidation of the β -carboxyl group of Asp101 with primary amines caused a decrease in the binding energy at subsites A and C, altering the initial rates of the lysozyme-catalyzed hydrolysis of the β -1,4 linkage of (GlcNAc)5 and the transglycosylation reaction.³⁰⁾ As demonstrated here, in RPL, Gly102 was replaced by Arg102. These results allow us to consider that the Asp101-Arg102 region of RPL has an almost identical net charge with

Table I. FLEXIBILITY OF AMINO ACID EXCHANGES ON LYSOZYME

Positions	3	15	19	21	34	40	41	55	68	73	77	79	84
Transitions	0	0	0	1	0	0	2	1	1	1	0	1	0
Transversions	1	4	0	0	1	1	2	0	0	0	1	0	1
Total	1	4	0	1	1	1	4	1	1	1	1	1	1
Total distance (A)	4	171	31	5	4	2	205	25	3	3	63	62	27
Total distance (B)	-70	5	-16	-13	-70	-14	5	-38	-35	-35	-16	-10	18

Positions	91	92	99	101	102	103	113	114	121	122	124	125
Transitions	0	1	1	1	2	0	0	1	3	1	1	1
Transversions	1	0	0	0	2	1	0	0	2	0	0	1
Total	1	1	1	1	4	1	0	1	5	1	1	1
Total distance (A)	2	6	6	65	122	63	31	64	329	6	16	3
Total distance (B)	-14	-2	-2	-6	67	-16	-10	-16	-72	-2	-1	-35

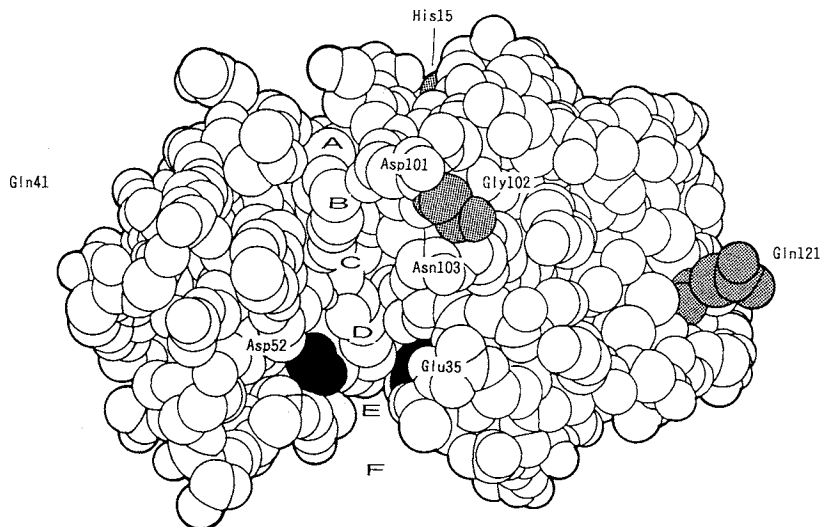


Fig. 5. Substituted Amino Acids on Stereo Structural Model of Hen Egg-white Lysozyme.

Subsite A–F are in the cleft located at the front of this model. Catalytic amino acids, Glu35 and Asp52 were indicated by solid circles. Hypervariable positions were indicated by shadowed circles. Position 41 was located at the back of this model.

that of the derivative of HEWL in which Asp101 is converted to Asn101.

We consider that RPL will be favorably used for elucidation of the role of the amino acid residue at position 101 and 102 (right-side loop region of subsite A–C) in lysozyme-catalyzed reactions.

Acknowledgments. The authors are indebted to The Kumamoto Zoological Park for supplying eggs.

References

- 1) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma, *Proc. Roy. Soc. B*, **167**, 378 (1967).
- 2) M. R. Pincus, S. S. Zimmerman and H. A. Scheraga, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2629 (1977).
- 3) M. R. Pincus and H. A. Scheraga, *Biochemistry*, **20**, 3960 (1981).
- 4) S. J. Smith-Gill, J. A. Rupley, M. R. Pincus, R. P. Carty and H. A. Scheraga, *Biochemistry*, **23**, 993 (1984).
- 5) T. Fukamizo, S. Goto, T. Torikata and T. Araki, *Agric. Biol. Chem.*, **53**, 2641 (1989).
- 6) T. Fukamizo, S. Goto, T. Torikata and T. Araki, *J. Biochem.*, **107**, 445 (1990).
- 7) A. M. Crestfield, S. Moore and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).
- 8) T. Araki, K. Kudo, M. Kuramoto and T. Torikata, *Agric. Biol. Chem.*, **53**, 2955 (1989).
- 9) J. Y. Chang, D. Brauer and B. Wittmann-Liebold, *FEBS Lett.*, **93**, 205 (1978).
- 10) C. Y. Yang, *Hoppe-Seyler's Z. Physiol. Chem.*, **360**, 1673 (1979).
- 11) T. Araki, M. Kuramoto and T. Torikata, *Agric. Biol. Chem.*, **54**, 2299 (1990).
- 12) J. N. LaRue and J. C. Speck, Jr., *J. Biol. Chem.*, **245**, 1985 (1970).
- 13) R. E. Canfield, *J. Biol. Chem.*, **238**, 2698 (1963).
- 14) J. Jollès, J. Jauregui-Adell, I. Bernier and P. Jollès, *Biochim. Biophys. Acta*, **78**, 668 (1963).
- 15) T. Araki, M. Kuramoto and T. Torikata, *Proc. Facul. Agric. Kyushu Tokai Univ.*, **7**, 81 (1988).
- 16) J. Jollès, I. M. Ibrahim, E. M. Prager, F. Shoentgen, P. Jollès and A. C. Wilson, *Biochemistry*, **18**, 2744 (1979).
- 17) M. Kaneda, I. Kato, N. Tominaga, K. Titani and K. Narita, *J. Biochem.*, **65**, 747 (1969).
- 18) E. M. Prager, N. Arnheim, G. A. Mross and A. C. Wilson, *J. Biol. Chem.*, **247**, 2905 (1972).
- 19) I. M. Ibrahim, E. M. Prager, T. J. White and A. C. Wilson, *Biochemistry*, **18**, 2736 (1979).
- 20) J. Jollès, E. Van Leemputten, A. Mouton and P. Jollès, *Biochim. Biophys. Acta*, **257**, 497 (1972).
- 21) J. L. Risler, M. O. Delorme, H. Delacroix and A. Henaut, *J. Mol. Biol.*, **204**, 1019 (1988).
- 22) R. M. Schwartz and M. O. Dayhoff, in "Evolution of Protein Molecules," ed. H. Matsubara and T. Yamanaka, Japan Scientific Societies Press, Tokyo, 1977, pp. 1–16.
- 23) T. J. White, G. A. Mross, E. F. Osserman and A.

- C. Wilson, *Biochemistry*, **16**, 1430 (1977).
- 24) Y. Ito, H. Yamada, S. Nakamura and T. Imoto, *J. Biochem.*, **107**, 236 (1990).
- 25) Y. Takagaki, A. Hirayama, H. Fujio and T. Amano, *Biochemistry*, **19**, 2498 (1980).
- 26) L. Adorini, M. A. Harvey, A. Miller and E. E. Sercarz, *J. Exp. Med.*, **150**, 293 (1979).
- 27) M. Z. Atassi and C. Lee, *Biochem. J.*, **171**, 419 (1978).
- 28) C. Lee and M. Z. Atassi, *Biochim. Biophys. Acta*, **495**, 354 (1977).
- 29) R. Jemmerson and E. Margoliash, *Nature*, **282**, 468 (1979).
- 30) T. Fukamizo, K. Hayashi and S. Goto, *Eur. J. Biochem.*, **158**, 463 (1986).
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