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Lysozyme [EC 3. 2. 1. 17 mucopeptide muramylhydrolase] can form stable complexes with glycol chitin (substrate) and its partially and completely hydrolyzed products under appropriate conditions. On forming the complex with the substrate, the ultraviolet absorption band of lysozyme shifts toward longer wavelengths (Hayashi et al., 1963). This red-shift of the spectrum arises from the burial of a specific tryptophan residue of lysozyme into the interior of the molecule. The burial of the tryptophan residue is accompanied by a conformational change of whole molecule, and not due to mere covering of the tryptophan residue with the substrate* (Hayashi et *al., 1964).*

The conformational changes accompanied by the formation of the enzyme-substrate cnmplexes have been observed with several enzymes by various physico-chemical techniques. Since the activity of a protease depends profoundly upon the conformation of the substrate proteins, the conformational changes in the enzyme molecule taking place on the formation of the enzyme-substrate complex can be followed with a change in the proteolytic digestibility of the enzyme molecule in the complex. Nirenberg and Jakoby (1960) reported that succinate semialdehyde dehydrogenase [EC 1. 2. 1. 16] had increased sensitivity for tryptic digestion in the presence of the substrate. This finding was regarded as an induction of the conformational change of the enzyme by its substrate.

It has long been known that the spectrum of a protein depends upon the environment of its chromophores ; for instance, the spectrum of tryptophan residues buried in an interior of the molecule is shifted toward longer wavelengths as compared to the residues accessible to medium. When such interior tryptophan residues are exposed to the

* In the present paper, "substrate" indicates only substrate of lysozyme, but not of proteases, unless noted elsewhere.

medium by denaturation or by cleavage of peptide bonds, the spectrum shifts toward shorter wavelengths (blue-shift). The difference spectrum of a denatured protein which originally contained buried tryptophan residues exhibits a negative peak around 293 mμ. The blue-shift of lysozyme which closely resembled the blue-shift of tryptophan was first observed by Donovan ***et al. (1958).*** Lysozyme contains four tryptophan residues accessible to the medium and two inaccessible residues which are located in an interior of the molecule (Hamaguchi and Kurono, 1963). Inada (1961) reported that one of three tyrosine residues of lysozyme behaved abnormally and was normalized in the presence of 4 M guanidine hydrochloride. The contribution of the abnormal tyrosine residue to the blue-shift of lysozyme can be excluded by measuring the intensity of the main negative peak at 293 mμ, because the blue-shift differrence spectrum arisen from the tyrosine residue shows no intensity at 293 mμ. Laskowski *et* ***al. (1956)*** applied first the difference spectrophotometry to the tryptic digestion of insulin. Sela and Anfinsen (1957) studied the peptic digestion of ribonuclease with the same method and Bigelow and Ottesen (1957) applied this method to observe the abnormal tyrosine residues in pepsin-inactivated ribonuclease. The proteolysis of lysozyme seems to be followed accurately by difference spectrophotometry, owing to its high content of tryptophan residues in the interior of the molecule.

The present paper deals with the digestibility of the lysozyme-substrate complex with proteases in connection with the conformational change.

Experimental

Materials

Lysozyme and glycol chitin were prepared as described previously (Hayashi ***et al., 1963).*** Variously hydrolyzed fractions of glycol chitin were isolated as follow : Lysozyme (600 mg) was added to 300 ml of an aqueous solution containing 6 g of glycol chitin. The mixture was kept at 50°C for 4 hrs, cooled by running water, and then passed through a column (4x30 cm) of carboxymethyl cellulose (II-form) to remove lysozyme. Ten volumes of acetone were added to the effluent to precipitate a fraction of comparatively large molecular products (F-H). The yield of F-H was 3.5 g. The supernatant was evaporated to 200 ml and 10 volumes of ethanol and 5 volumes of ether were added. The precipitate was called F-L (0.6 g). The polymerization degree of F-H and F-L were 11.0 and 5.1, respectively.

Three times recystallized pepsin was supplied by National Biochemi-

cal Co., pronase (45,000 P.U.K. /g) from Kaken Co. and trypsin (1:250) from Difco, respectively. All reagents were of analytical grade.

Methods

The rate of proteolysis of lysozyme or lysozyme-substrate complexes was followed by difference spectrophotometry, titration (Jacobsen *et al., 1957)* and precipitation with trichloroacetic acid (TCA).

A Beckman Model DB spectrophotometer equipped with a thermostat unit was used for difference spectrophotometry. Two cells were placed in the reference compartment ; one containing 3 ml of 0.1 % lysozymc ($OD_{280}=2.5$) and the other 3 ml of a buffer solution. The contents of cells in the sample compartment were first the same as those in the reference compartment. The cells were allowed to stand for 30 min in the temperature-controlled compartments, and a differece spectrum was recorded to correct the imperfect matching of cells. This difference spectrum was subtracted from that measured on the reaction mixture. Then, 0.3 ml of proteasc solution was added to the cell containing buffer solution in the sample compartment and 0.3 ml of buffer solution to the other cell containing lysozymc solution. Finally. 0.3 ml of protease solution was added to the cell containing lysozyme solution in the reference compartment, and reaction time was measured from this point. It was already observed that the spectrum of digested lysozyme shifted toward shorter wavelengths owing to the exposure of buried tryptophan residue to the medium, resulting in the blue-shift difference spectrum with negative peak around 290 mμ. Since, in the present experiment, the cell containing reaction mixture was placed in the reference compartment, the difference spectrum was recorded as the red-shift difference spectrum with positive peaks. The main peak at 293 mμ of the difference spectrum, ΔOD_{293}, in absorbancy units was plotted as a function of the reaction time. In the case of proteolysis of the lysozyme-substrate complex, a solution containing 0.1 % lysozme and 0.1 % substrate was used instead of the lysozyme solution. The amount of the lysozymc-substrate complex was estimated by measuring the intensity of the difference spectrum of the complex at 293 mμ referred to lysozymc alone at the same concentration. To avoid confusion, the systems used for measurement of the difference spectrum are listed in Table I, and the difference spectrum of the lysozyme-substrate complex referred to lysozyme alone, measured by system 1, was distinguished by the symbol (C) from those measured by the other systems.

The titration was carried out with a pH-statt, Kadiometer TTT-1. Copenhagen, equipped with a temperature regulation cell using 0.1 N HC1. The amount of HC1 consumed was converted into the number

Table 1. Measuring systems for difference spectra.

No. of system	Compartment	Cell[a]	Type of diff. spectra	Origin
1. Formation of ES-complex	Sample side	(E+S+B)(B)	Red-shift[b]	Burial of Trp in more dense region
	Reference side,	(E+B)(S+B)		
2. Proteolysis of ES-complex	Sample side	(E+S+B)(P+B)	Red-shift[c]	Exposure of Trp from dense region to medium
	Reference side	(E+S+P+B)(B)		
3. Proteolysis of lysozyme	Sample side	(E+B)(P+B)	Red-shift"	Same as above
	Reference side	(E+P+B) (B)		

a): Parentheses indicates the content of a cell.
b): This was denoted by (C) in the text.
c): The reaction mixture was filled in a cell at the reference compartment, so that the blue-shift was observed as the red-shift.
E, S, P and B represent lysozyme, substrate, protease and buffer solution.

of peptide bonds cleaved.

One tenth per cent lysozyme solution became a homogeneously turbid suspension on addition of TCA to 5 %. The turbidity of the suspension was measured as the transmittance of light at 660 mμ and the rate of proteolysis was determined from this value.

Crystals of the lysozyme-substrate complex were isolated according to the method described previously (Hayashi et al., 1968). The concentration of lysozyme was determined spectrophotometrically using $\varepsilon_{280}=38{,}700$ (Foss, 1961).

Results

Crystals of the complex

The yield of crystals of the lysozyme-substrate complex was 58 % based on the total amount of lysozyme present. The sugar content of the crystals of the complex determined by the method of Elson and Morgan (1933) was 8.3 % by weight. In 0.1 M phosphate buffer at pH 5.6, the intensity of the difference spectrum (C) of 0.1 % crystallized complex* was 0.07 at 293 mμ. This value corresponds to 70 % of the intensity of the difference spectrum (C) arising from the mixture of

* The concentration of the complex indicates that of the lysozyme moiety.

the same amount of lysozyme and substrate. This fact indicates that about 30 % of total lysozyme in the crystallized complex was free in solution due to the equilibrated dissociation of the complex.

Proteolysis with pepsin (Difference spectrophotometry)

(1). The rate of proteolysis at pH 2.5 and 45°C is shown in Fig. 1. The intensity of the difference spectrum at 293 mμ, ΔOD_{293}, arising from the complete proteolysis of lysozyme alone was found to be 0.27. The rate of proteolysis of the lysozyme-substrate complex with intact glycol chitin was smaller than that of lysozyme alone. The extent of proteolysis of the complex with F-L was much smaller than

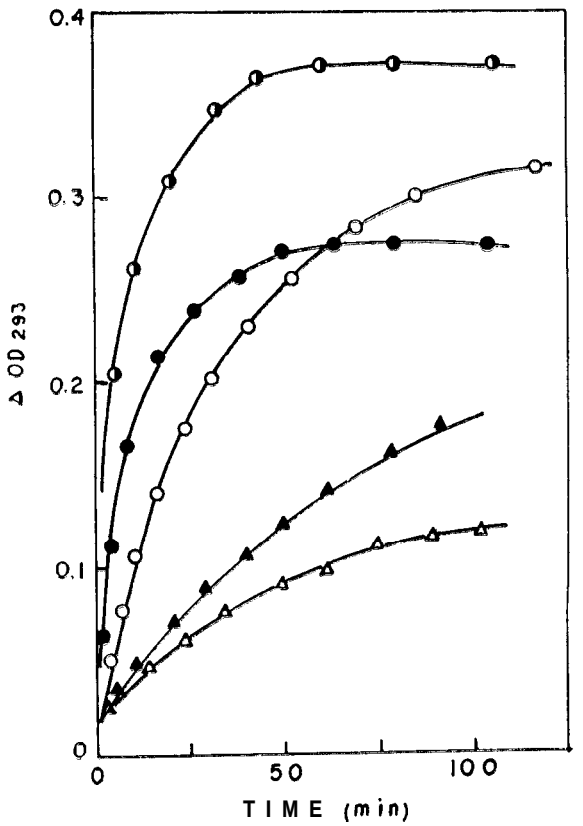

Fig. 1. Proteolysis of lysozyme-substrate complex with pepsin. Concentrations of lysozyme and pepsin were 0.1 % and 0.01 %, respectively, Proteolysis was carried out in 0.1 M citrate buffer, pH 2.5 at 40°C, and followed by difference spectrophotometry.
● : Lysozyme, ◑ : calculated value, ○ : complex with intact glycol chitin, ▲ : complex with F-H, △ : complex with F-L.

those of lysozyme alone and of the complex with intact glycol chitin. The rate of proteolysis depends strongly- upon the molecular size of substrates; the smaller the molecular size, the smaller the rate was observed. Two tryptophan residues in lysozyme are buried in the interior of the molecule and are solvent-inaccessible (Hamaguchi and Krono, 1963). The exposure of these residues to a medium by the cleavage of peptide bonds gave $\Delta OD_{293}=0.27$ at 0.1 % lysozyme concentration. In the case of the complex, three tryptophan residues located in the interior of the molecule and the exposure of these residues should giv e $\Delta OD_{293}=0.41$. Actually, ΔOD_{293} (C) of 0.1 % lysozyme-substrate complex was found to be 0.098 at pH 2.5 and 0.11 at pH 5.6, where all lysozymc forms the complex. Therefore, 90 % of of lysozymc formed the complex and 10 % was free al pII 2.5. Consequently, the

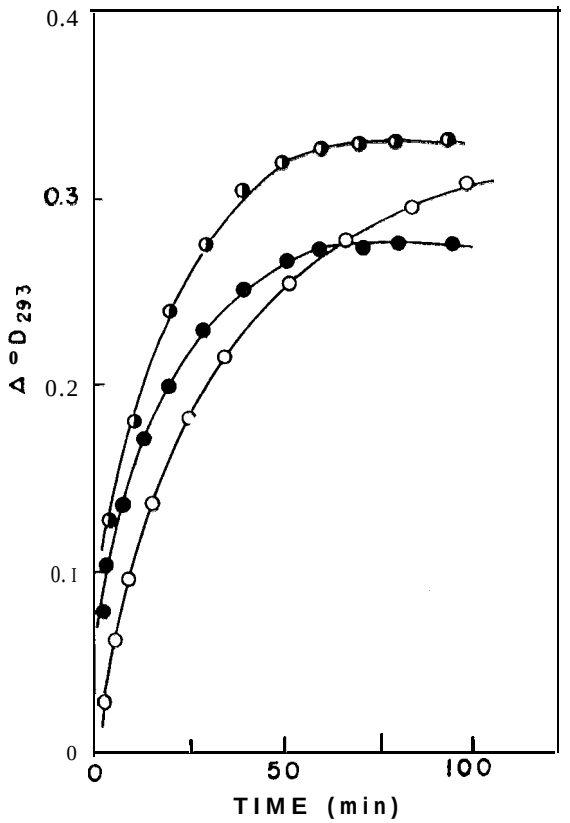

Fig. 2. I'roteolysis of lysozme and of crystallized complex with pepsin in 0.1M citrate buffer, pH 2.5 and 45°C. Concentration of lysozyme was 0.1 %.
● : Lysozyme, ○: crystallized complex, ◑: calculated value,

value of ΔOD_{293} at the complete proteolysis of 0.1 % lysozyme-substrate complex was calculated to be 0.368 = (0.27+0.098). If the rate of proteolysis of the complex is the same as that of lysozyme alone, the value of ΔOD_{293} measured in the process of proteolysis of the complex should be one which is obtained by multiplying the factor 1.37 = (0.368/0.270), to the value observed on lysozyme alone at the same reaction time. The values thus calculated were plotted in the figures as the calculated value. The value measured on the complex must be compared with the calculated value.

(2). It is characteristic that the complex with substrate of low molecular size shows a great resistance to peptic digestion. In this case, there is a possibility that the free substrate not participating in the formation of the complex may inhibit directly the action of pepsin. The use of crystallized complex seems to determine the above possibility, because the crystals consists of 1 mole of lysozyme and 1 mole of F-L. The protcolysis of the crystallized complex dissolved in 0.1 M citrate buffer is shown in Fig. 2.

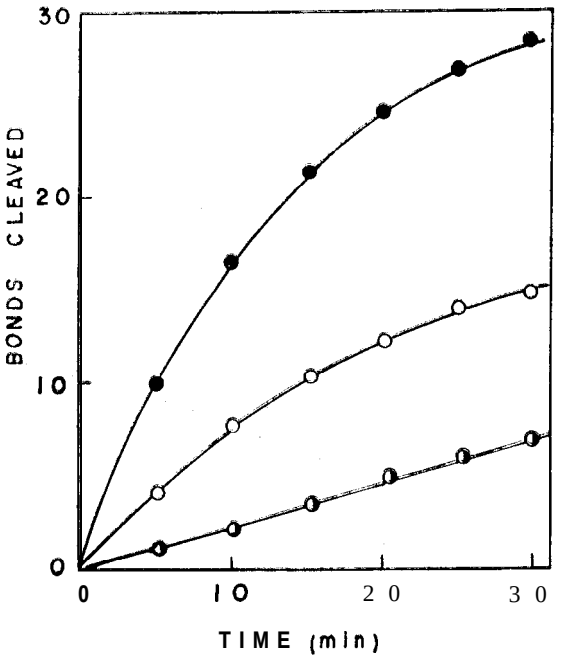

Fig. 3. Proteolysis with pepsin followed by titration at pH 3.3 and 47°C. The reaction mixture consisted of 3 ml of 0.1% lysozyme or complex and 1 ml of 0.2% pepsin.
● : Bonds cleaved per 1 mole lysozyme, ◡ : bonds cleaved per 1 mole complex with F-II, ◑ : autolysis of pepsin.

The calculated value plotted in Fig. 2 was obtained from the value of ΔOD_{293} (C) of 0.1 % crystallized complex. When ΔOD_{293} (C) (=0.055) of 0.1 % crystallized complex dissolved in a citrate buffer was added to the maximum value of ΔOD_{293} of complete proteolysis of lysozyme alone, the total value from the complete proteolysis of the complex was calculated to be 0.325. The factor, 1.2=(0.325/0.270), was multiplied by the value of ΔOD_{293} at each reaction time, and the calculated value was obtained. The rate of proteolysis of the crystallized complex dissolved in 0.1 M citrate buffer was small at the initial stage of the reaction. Then the curve gradually approached the calculated value. The features of over-all proteolysis were quite different from that observed on the complex with F-L (see Fig. 1). This discrepancy will be discussed later.

Proteolysis with pepsin (Titration)

(1). The proteolysis at pH 2.5 and 47°C followed by titration with

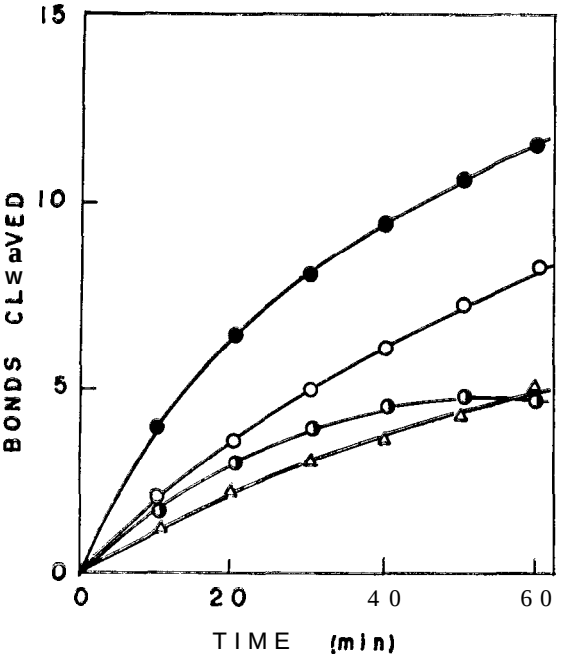

Fig. 4. Effect of molecular size of substrate on the rate of proteolysis of complex with pepsin, Proteolysis was carried out at pH 2.5 and 30°C. Concentration of lysozyme or complex was 0.5 % and pepsin was 0.01 %.
● : Lysozyme, ○: complex with intact glycol chitin,
△: complex with F-L, ◑: autolysis of pepsin.

0.1 N HCl is shown in Fig. 3. A titration owing to the autolysis of pepsin, also measured. The proteolysis at pH 3.5 was similar to that at pH 2.5.

(2). The lysozyme-substrate complex formed with intact glycol chitin was more easily proteolyzed than those with F-L in agreement with the result by difference spectrophotometry as shown in Fig. 4.

(3). The presence of F-L delayed very slightly proteolysis of lysozyme oxidized with equimolar N-bromosuccinimide (NBS) as shown in Fig. 5. Lysozyme oxidized with equimolar NBS has no activity, and did not show the difference spectrum (C). This suggests that there is no interaction between the oxidized lysozyme and the substrate.

Proteolysis with pepsin (TCA method)

Lysozyme solution (0.1 %) became a homogeneously turbid suspen-

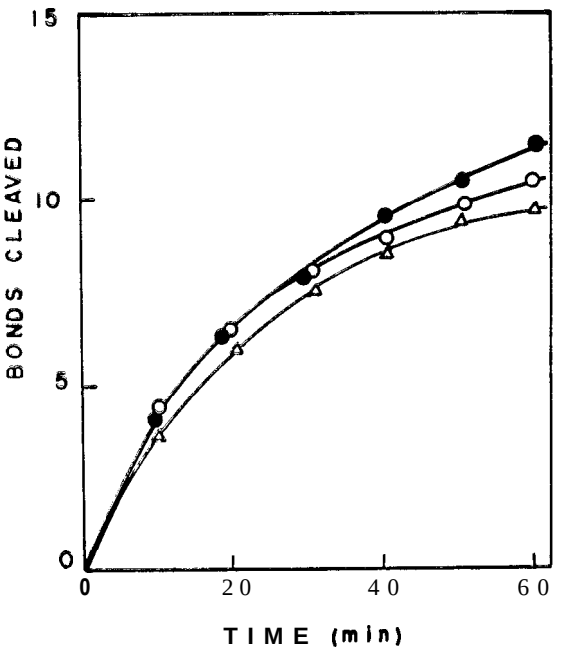

Fig. *5.* Proteolysis of NBS-oxidizd lysozyme with pepsin followed by titration at pH 2.5 and 30°C. Concentrations were the same as those in Fig. 4.
● : Lysozyme, ○ : NBS-oxidized lysozyme,
△ : NBS-oxidized lysozyme plus *0.5* % F-L.

sion with addition of TCA to a final concentration of 5 %. Proteolysis of lysozyme and the complex with F-L at pH 2.5 and 47°C measured by TCA method is shown in Fig. 6.

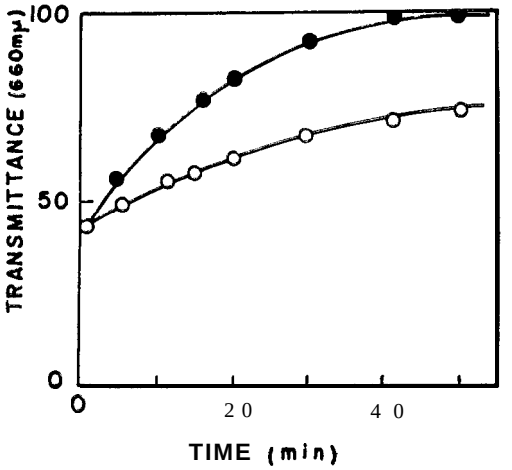

Fig. 6. Proteolysis of lysozyme and of complex followed by TCA method in 0.1 M citrate buffer, pH 2.3 at 47 C. Reaction mixture consisted of 30 ml of 0.1 % lysozyme or complex and 2 ml of 0.2 % pepsin.
● ■ Lysozyme, ◡ : complex.

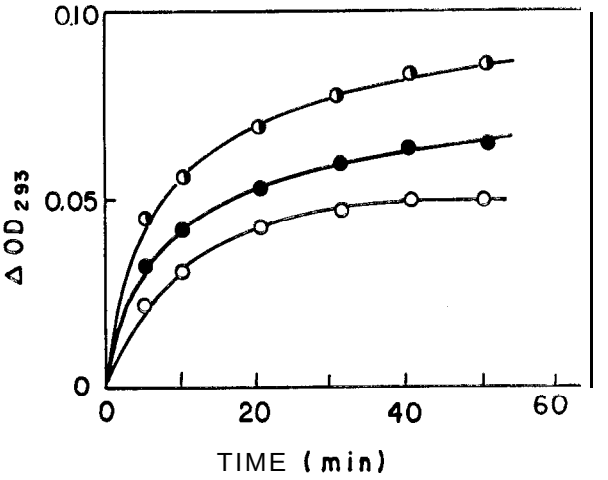

Fig. 7. Proteolysis with pronase followed by differencespectrophotometry in 0.1 M phosphate buffer, pH6.0 at 45 C. Reaction mixture consisted of 3 ml of 0.1 % lysozyme or complex and 0.3 ml of 0.1 % yronase.
● : Lysozyme, ◯ : complex with F-L, ◑ : calculated value.

Proteolysis with pronase (Difference spectrophotometry)

The result obtained at pH 6.0 and 45°C by difference spectrophotometry is shown in Fig. 7. Ninety five per cent of the total lysozyme was in the form of the complex with F-H at pH 6.0.

Proteolysis with trypsin (Difference spectrophotometry)

Tryptic digestion was followed by difference spectrophotometry. The result obtained at pH 8.0 and 45°C is shown in Fig. 8. Proteolysis ceased within 30 min under experimental conditions. The initial rate of proteolysis of the complex was considerably smaller than that of lysozyme alone as noted with other proteases.

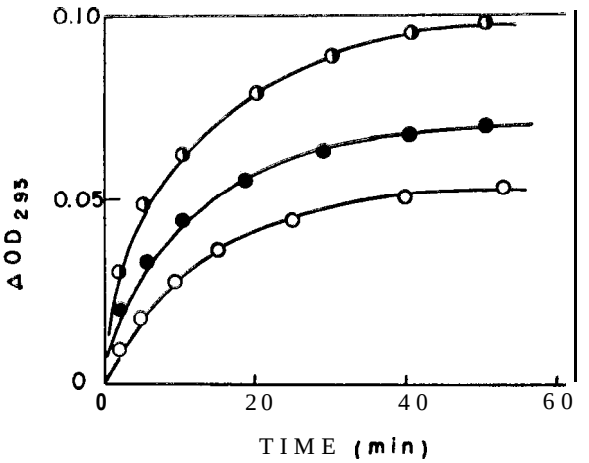

Fig. 8. Proteolysis with trypsin in 0.1 M phosphate buffer, pH 8.0 at 45°C. Reaction mixture consisted of 3 ml of 0.1 % lysozyme or complex and 0.3 ml of 0.1 % trypsin.
●: Lysozyme, ○: complex with F-L, ◑: calculated value.

Discussion

The polymerization degree of the final product, F-L, derived from the complete hydrolysis of glycol chitin with lysozyme was found to be 5.1 on an average. The sugar content of the crystallized complex was 8.3 %. Assuming that 1 mole of lysozyme combines with 1 mole of sugar at the active site, the polymerization degree of sugar moiety in the complex was calculated to be 4.9. Since F-L whose polymerization degree is 5.1 could not bc hydrolyzed further with lysozyme, it can be concluded that 1 mole of crystallized complex contains 1 mole of F-L.

When the lysozyme molecule was completely digested with protease and two tryptophan residues originally buried in the interior of the molecule were exposed to a medium, a blue-shift difference spectrum was observed. The intensity of this spectrum at 293 mμ with system 3 was found to be 0.27 at 0.1 % lysozyme solution. In the case of lysozyme-substrate complex, there are three tryptophan residues buried in the interior of the molecule. After complete proteolysis of the complex, the intensity of difference spectrum at 293 mμ, ΔOD_{293}, referred to non-proteolyzed complex should be 0.41. The measured value with system 2, however, was 0.368 at pH 2.5 and 0.380 at pH 5.6 where all lysozyme molecules are in the form of the complex. This fact implies that the tryptophan residues buried in the interior of the molecule on formation of the complex is located in a different environment from those occupied by tryptophan residues originally buried. ΔOD_{293} (C) of 0.1 % lysozyme-substrate complex was 0.110 at pH 5.6 and 0.095 at pH 2.5. Eighty eight per cent of lysozyme present was in the form of the complex at pH 2.5 and other 12 % was free which seems to be hydrolyzed with the same rate as lysozyme alone. The contribution of this free lysozyme need not be considered as far as the rate of proteolysis of the complex was compared to the calculated value.

Table 2. Dependence of the formation of lysozyme-substrate complex on pH.

pH	ΔOD_{293} (C)[a]	Relative amount of ES-complex	Relative activity[b]
2.5	0.094	88	50
3.3	0.104	95	75
4.5			100
5.6	0.110	100	60
6.0	0.104	95	50
8.0	0.080	73	25
2.5"	0.055	50	

a) Concentration of lysozyme was 0.1 %.
b) Measured by viscometry.
c) Crystallized complex.

Although F-L can be hydrolyzed no further with lysozyme, it can still form the complex with lysozyme which shows the same difference spectrum (C) as the complex with intact glycol chitin. This type of the complex has been defined as nonproductive complex. A complex with a substrate having low molecular weight shows much resistance to digestion with proteases. Since the lysozyme-substrate

complex is completely formed under appropriate conditions by mixing the equal weights of lysozyme and substrate regardless of the molecular size of the substrate, then smaller the substrate, the larger the number of substrate molecule is free in the solution. It is, therefore, possible that the free substrate inhibits directly the action of proteases. In order to examine this possibility, the proteolysis of the crystallized complex was carried out. The crystallized complex consists of 1 mole of lysozyme and 1 mole of F-L. When the crystallized complex was dissolved in 0.1 M citrate buffer at pH 2.5, about 50 % of lysozyme and the substrate became free by equilibrated dissociation. The amount of free substrate, however, was negligibly small in comparison with the amount of free substrate on mixing equal amounts of lysozme and F-L. As can be seen in Fig. 2, the rate of proteolysis of the crystallized complex was very large and comparable to that of the complex with intact substrate. This fact seems to indicate the direct inhibition of proteases by free F-L. A more reliable experiment was carried out by titration method on the proteolysis of NBS-oxidized lysozyme in the presence of F-L. The possibility of direct inhibition of pepsin by F-L was entirely excluded as seen in Fig. 5. The high sensitivity of the crystallized complex to proteolysis should be explained in some other way.

At the initial stage of the reaction, as seen in Fig. 2, the rate of peptic digestion of the crystallized complex was very low than that of lysozyme alone. The rate increased with increase in the reaction time. This fact will be explained by assuming that the substrate in the crystallized complex was easily split off following the cleavage of a few peptide bonds of lysozyme, and the partially digested lysozyme molecule free of the substrate was then digested at the same rate as lysozyme alone. Since the complex with F-L in Fig. 1 was equilibrated with a large molar excess of substrate, the leaving of the substrate from partially digested complex seemed to be inhibited, consequently the proteolysis may be delayed markedly. In the cases of the complexes with intact glycolchitin and the crystallized complex, the number of free substrate was very small. This might cause the relatively high rate of protcolysis of these complexes. The dependence of the rate of proteolysis of the complexes on the molecular sized of the substrate, therefore, seems to be partially attributable to the equilibrium between substrate molecules in complex and in the free state.

The autolysis of pepsin (0.0125 %) resulted in the consumption of a considerable amount of 0.1 N HCl in titration method. The collision between pepsin molecules should be strongly diminished by the presence of lysozyme or the complex (10 times as concentrated as pepsin on a molar basis) as substrates, and as the result the contribution

of autolysis of pepsin to the titration seems to become negligible. Experimentally, the correction of the measured rate for the autolysis of pepsin was not done, because the assumption that the contributions of autolysis of pepsin are the same and negligible for both cases, proteolyses of lysozme alone and the complex, is permissible. Alarge resistance of the complex to peptic digestion, at all events, was also evidenced by the titration method which gives directly the number of bonds cleaved.

Although the three methods used in the present study measure different parameters, the results obtained by these methods were in quite good agreement. Furthermore, the use of trypsin and pronase gave similar results to those found with pepsin, independent of their substrate-specificities. This fact suggests that not only the specific bonds but some bonds were uniformly protected from the action of proteases by the binding of the substrate to the lysozymc molecule.

Recently, Blake et al. (1965) reported that the inhibitor N, N′-diacetylchitobiose is bound in the cleft of the lysozyme molecule in which the binding site was involved. In the binding site, there are 24 residues (19 % of total 129 residues) of which side chains interact apparently with the inhibitor molecule. The number of residues participating in the binding of substrate is surprisingly larger than that considered vaguely so far. In the case of iysozyme-substrate complex, it seems reasonable that the great resistance of the complex to the proteolysis was caused by mere binding of substrate at the binding site. However, the decrease in digestibility of the complex attained to about 50 %, and this value is too large to explain changes in digestibility by only the binding of the substrate. The present results suggest that the resistance of the complex to proteolysis was also derived from the conformational changes of side chains in the lysozyme molecule on the complex formation in solution.

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