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72. Otomatsu Hoshino : Studies on the Constitution of Muco-complex from *Micrococcus lysodeikticus*. V.[1] On the Structure of the Degradation Products with Lysozyme.

(*Faculty of Pharmaceutical Sciences, University of Tokyo*[*1])

Several investigations on enzymatic hydrolysis products of muco-complex obtained from *M. lysodeikticus* are encountered. Thus, Hawthorne[2] detected ninhydrin-positive products in dialysable fraction of the hydrolysate. Schütte and Krisch[3] obtained non-dialysable, high-molecular products in ca. 30~40% yield after digestion of Meyer's muco-complex with lysozyme while they could not detect either free glucose or acetylglucosamine in the dialysable fraction.

On the lysozyme-digested products of muco-complex from cell walls of the same bacteria, Salton[4] reported both a dialysable fraction in 50% yield containing no free glucose or amino acids but low molecular "small fragment" detectable with aniline hydrogenphthalate or Morgan-Elson coloration and a non-dialysable fraction consisting of a mixture of high molecular products having molecular weight of the order of 10,000~20,000. Further, he stated that the "small fragment" was an acidic disaccharide (dimer) which gave, on acid hydrolysis, both glucosamine and an amino sugar similar to Strange's muramic acid.[5]

In addition to the literature cited above, a few investigations[*2] have been reported on the structure of lysozyme hydrolysate of muco-complex from the same bacteria.

Previous papers of this series reported the isolation of an electrophoretically pure muco-complex (fraction GIIs) from *M. lysodeikticus* which was digestable with lysozyme and its physicochemical properties[6,7] as well as its chemical nature that it contained amino sugars, both muramic acid and glucosamine, in a ratio of 1:1.[8] Further, in the preceding paper[1] fractionation and properties of lysozyme hydrolysates of both fractions GII and DNP-GII were described.

This paper deals with a more precise observations of the partial structure of lysozyme hydrolysis product based on hypoiodite and periodate oxidations.

Experimental

The products from lysozyme hydrolysis described in the preceding paper,[1] obtained in relatively high yield, were investigated. The MeOH-soluble part (named SLM) of lysozyme digest was fractionated by a charcoal-Celite column chromatography and the fractions obtained from the eluates by successive elution with 30%, 40~50%, and 50% EtOH containing 2% of pyridine were named E. 1, E. 2, and E. 4, respectively. On paper chromatography combined with paper electrophoresis, E. 1, E. 2, and E. 4 respectively gave a single spot, SL-1, SL-2, and SL-4. The MeOH-insoluble fraction was named SLW. The following experiments were performed for E. 1. E. 2, E. 4, and SLW.

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*1 Hongo, Bunkyo-ku, Tokyo (星野乙松).

*2 Recently, Park, *et al.*[9] proposed a speculative structure for the muco-complex as the substrate of lysozyme.

1) Part IV : This Bulletin, 8, 405(1960).
2) J. R. Hawthorne : Biochim. et Biophys. Acta, **6**, 94(1950).
3) E. Schütte, K. Krisch : Z. physiol. Chem. Hoppe-Seyler's, **311**, 121(1958).
4) M. R. J. Salton : Biochim. et Biophys. Acta, **22**, 495(1956).
5) R. E. Strange, F. A. Dark : Nature, **177**, 186(1956); R. E. Strange : Biochem. J., **64**, 23p(1956).
6) Part I. S. Akiya, O. Hoshino : Yakugaku Zasshi, **77**, 777(1957).
7) Part II : This Bulletin, 8, 395(1960).
8) Part III : *Ibid.*, 8, 399(1960).
9) W. Brumfitt, A. C. Wardlaw, J. T. Park : Nature, **181**, 1783(1958).

Hypoiodite Oxidation of Lysozyme Degradation Products—5.10 mg. of E. 1 was dissolved in 0.5 cc. of 0.1*N* I_2 containing 0.5 cc. of *N* NaOH. After standing for 60 min. at room temperature, the solution was titrated with 0.01*N* $Na_2S_2O_3$. E. 2 (10.90 mg.), E. 4 (10.80 mg.), and SLW (13.08 mg.) were treated similarly. N-Acetylglucosamine (2.20 mg.) was used for the control.

Electrometric Titration of Degradation Products—Each of E. 1 (5.70 mg.), E. 4 (6.25 mg.), and SLW (6.70 mg.) was dissolved in 1 cc. of water and titrated electrometrically with 0.01*N* NaOH.

Periodate Oxidation—E. 1 (6.39 mg.) was added to 1 cc. of 0.05*M* $NaIO_4$ and the solution was adjusted to 5 cc. with water, and titrated with 0.01*N* As_2O_3. E. 4 (20.20 mg.), SLW (40.56 mg.), and GII (61.00 mg.) was each added to 2 cc. of 0.05*M* $NaIO_4$. The solution was adjusted to 10 cc. with water and consumption of periodate was measured as above. For control N-acetylglucosamine, α-methylglucoside, and dextrin were used.

Periodate Oxidation of Lysozyme Digest of GII—The solution of GII (16.16 mg.) and lysozyme (0.2 mg.) dissolved in 10 cc. of water was allowed to stand for 16 hr. at 37°. To this solution, 2 cc. of 0.05*M* $NaIO_4$ was added and the mole of periodate consumed was measured at intervals.

Properties of E. 1—Lyophilized E. 1 came as a very hygroscopic white powder, soluble in H_2O and MeOH, slightly soluble in EtOH and Me_2CO, and insoluble in Et_2O. Attempts to crystallize this disaccharide from several solvents were unsuccessful. Lyophilized E. 1 decomposed on heating to above 128~130° (evolution of gas); $[\alpha]_D^{25}$ +22.0°(c=1.0, H_2O). *Anal.* Calcd. for $C_{19}H_{32}O_{13}N_2 \cdot 2H_2O$ (Disaccharide of N-acetylglucosamine and N-acetylmuramic acid) : C, 42.85; H, 6.77; N, 5.26. Found: C, 41.92; H, 6.80; N, 5.32.

Coloring Substances in the Elson-Morgan Reaction of E. 1—1) Changes of color during hydrolysis of E. 1 : 2.2 mg. of E. 1 was hydrolysed with 5 cc. of *N* HCl on a boiling water bath and after 10, 30, 60, or 90 min., 1 cc. each of the hydrolysate was drawn and the color developed was measured by the method of Elson-Morgan.

2) 4.0 mg. of E. 1 was dissolved in 2 cc. of water, 0.5 cc. of *N* NaOH, 0.5 cc. of 0.1*N* I_2, and 1 cc. of water were added to the solution and the mixture was allowed to stand at room temperature for 2 hr. Two cc. of the reaction mixture was passed through a column (10×1 cm.) of Dowex-50 and washed with water to obtain 10 cc. of eluate. One cc. of this eluate was hydrolysed with 1 cc. of 2*N* HCl and hexosamine in the hydrolysate was determined by the method of Elson-Morgan.

3) E. 1 (9.1 mg., 5 cc.) was oxidized with periodate, 1 cc. of the reaction mixture was decationised with Dowex-50, and hydrolysed with 2*N* HCl. Hexosamine in the hydrolysate was determined by the method of Elson-Morgan.

Substances produced by Periodate Oxidation of E. 1—1) Estimation of formic acid : A solution of 10.0 mg. of E. 1 dissolved in 5 cc. of 0.05*M* $NaIO_4$ and 5 cc. of water was allowed to stand at room temperature. To each 1 cc. of the solution, 1 cc. of 5% ethylene glycol was added at intervals and formic acid produced was titrated with 0.01*N* NaOH. For control, glucose (5.0 mg.) and N-acetylglucosamine (5.0 mg.) were used.

2) Estimation of formaldehyde : Oxidation conditions were the same as that of (1). Formaldehyde produced was measured colorimetrically by chromotropic acid method.[10,11]

Sodium Borohydride Reduction of E. 1 and Periodate Oxidation of the Product (E′. 1)—To a solution of 30.0 mg. of E. 1 dissolved in 1 cc. of water, 5 mg. of $NaBH_4$ was added and allowed to stand overnight at room temperature. The reaction mixture was linearly streaked on Toyo Roshi No. 27 (40×30 cm.), 15 cm. apart from the cathode, and submitted to zone electrophoresis (pyridine-acetic acid buffer of pyridine 10 cc., AcOH 2 cc., BuOH 40 cc., H_2O 948 cc.; pH 5.8, 15~25 v/cm., 1.5~2.0 mA/cm., 3 hr.). Then margins from both edges of the filter paper were cut off and location of the reduced product (E′. 1) was detected with periodate-benzidine spray. The part of the paper containing E′. 1 was cut out from unsprayed paper and the aqueous eluate from the cutting was lyophilized. The yield of reduced product (E′. 1) was 13.8 mg. (46.0%). Periodate oxidation and estimation of formic acid and formaldehyde were performed as described in the case of E. 1.

Results

Properties of Hydrolysis Products

(1) Hypoiodite Oxidation—After digestion of GII with lysozyme, several fractions obtained were oxidized with hypoiodite to convert their reducing groups into carboxyl groups. On calculation of their molecular weights from iodine consumption in above reaction, that of E. 1 was 510 which agreed approximately with that of disaccharide composed of 1 mole each of N-acetylglucosamine (N-AcGm) and N-acetylmuramic acid (N-AcMA). Calcd. for $C_{19}H_{32}O_{13}N_2 \cdot 2H_2O$: mol. wt., 532. The molecular weight of E. 2 was 1,006 (tetrasaccharide consisting of 2 moles each of N-AcGm and N-

10) S. A. Barker, A. B. Foster, M. Stacey, J. M. Webber : J. Chem. Soc., **1958**, 2218.
11) J. F. O'Dea, R. A. Gibbons : Biochem. J., **55**, 580(1953).

AcMA). From the acid hydrolysate of both E. 1 and E. 2, no amino acid or glucose but glucosamine and muramic acid were detected on combined paper chromatography.

(2) Electrometric Titration and Infrared Spectra—The electrometric titration curves of E. 1, E. 4, and SLW are shown in Fig. 1 which give evidence of acidic compounds with neutralization equivalent of 450~600 for each fraction. The infrared spectra of E. 1, E. 4, SLW, and GII show similar absorptions corresponding to $-NHCOCH_3$ at 1545 and 1637 cm^{-1} and -COOH at 1720 cm^{-1}.

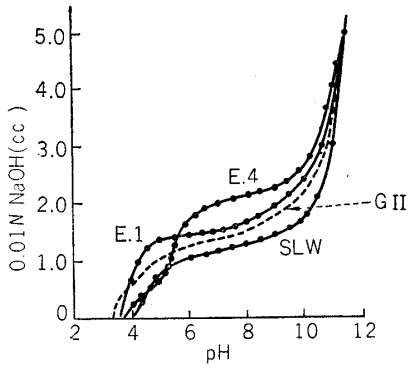

Fig. 1.

Electrometric Titration Curves of Lysozyme-Digested Fragments

(3) Periodate Oxidation—Fig. 2 shows the result of periodate oxidation of E. 1, E. 4, SLW, and GII. Also as shown in Fig. 2, GII consumed ca. 1.0~1.1 moles of the reagent per molecular weight of 1,000 after 24 hr. After digestion of GII with lysozyme, the periodate consumption amounted to 1.8 moles after 24 hr. Above observation, together with the fact that the consumptions by E. 1. E. 4, and SLW were greater than that by GII, indicates gradual increase of reducing groups after their enzymatic hydrolysis.

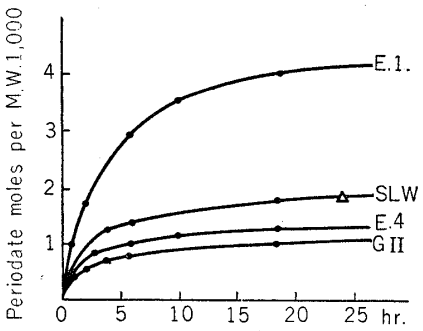

Fig. 2.

Periodate Consumption of E. 1, E. 4, SLW, and GII

△ Periodate consumption of GII after digestion with lysozyme

Several properties of GII and its lysozyme hydrolysates are given in Table I. The smallest hydrolysis product from GII with lysozyme, E. 1, obtainable in paper-electrophoretically pure state and in a good yield, was assumed to be an acidic disaccharide composed of AcGm and AcMA, probably the same compound as Salton's "small fragment,"[4] and this was used for further investigation.

Table I. Several Properties of GII and its Lysozyme Hydrolysates

Compd.	Mol. wt. detd. by hypoiodite	Consumption of periodate* mole/mol. equiv.	Neutral. equiv.	Component
E. 1	510	2	475	Ac-Gm, Ac-MA
E. 2	1,006	—	—	Ac-Gm, Ac-MA
E. 4	1,300	2	391	Ac-Gm, Ac-MA, Peptide
SLW	>1,600	3	609	Ac-Gm, Ac-MA, Glucose Peptide (Ala, Gly, Glu, Lys)
GII	ca. 100,000	1.0~1.1 (per mol. wt. 1,000)	626	〃

* after 24 hr.

Elson-Morgan Reaction of E. 1

(1) Periodical Variation during Acid Hydrolysis—Absorption spectra of colored complexes in the Elson-Morgan reaction for aliquots taken at intervals in acid hydrolysis of E. 1 were measured

and the results are given in Fig. 3. The maximum absorption at 540 mμ before hydrolysis of E. 1 gradually showed a hypsochromic shift to 510 mμ. Thus, the original absorption at 540 mμ is due to the terminal reducing group.

(2) Absorption Spectra after Hypoiodite or Periodate Oxidation—Fig. 4 shows the same kind of curves by Elson-Morgan coloration after oxidation of E. 1. Curve 1 was obtained from the mixed hydrolysate of E. 1 and curve 2 was obtained after hypoiodite oxidation, followed by hydrolysis of E. 1; that is, this curve is caused by a componental amino sugar liberated from E. 1, the aldehyde group of which takes part in glycosidic linkage before hydrolysis.

In Fig. 4, the authentic glucosamine gave the corresponding curve (curve A) with a maximum at 530 mμ and the maximum in curve 2 is at 510 mμ.[5] These observations together with the results shown in Fig. 3 suggest that (i) in the original E. 1, the reactive reducing group for Elson-Morgan coloration is present in glucosamine residue of the disaccharide and that (ii) migration of the maximum to 510 mμ after hydrolysis of E. 1 should show the increase of new reactive reducing group for the coloration, which is probably involved in muramic acid and which was linked to the glucosamine part before the hydrolysis.

Curve 3 in Fig. 4 represents the Elson-Morgan coloration after periodate oxidation of E. 1 and is very similar to curve 2. On periodate oxidation, 1 mole of E. 1 consumed about 2 moles of the reagent after 24 hours. From these results, it is evident that by this oxidation only the reactive group for coloration involved in muramic acid part remained intact.

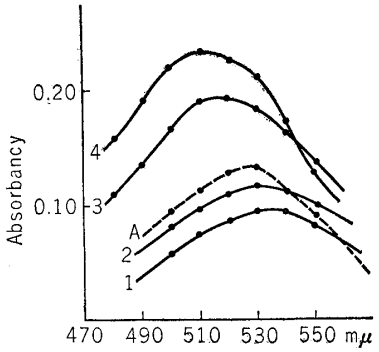

Fig. 3. Absorption Spectra of E. 1 in Elson-Morgan Reaction during Acid Hydrolysis (1*N* HCl, 100°)

Curve 1 : before hydrolysis
〃 2 : after 10 min.
〃 3 : after 30 min.
〃 4 : after 60 min.
〃 A : glucosamine for control

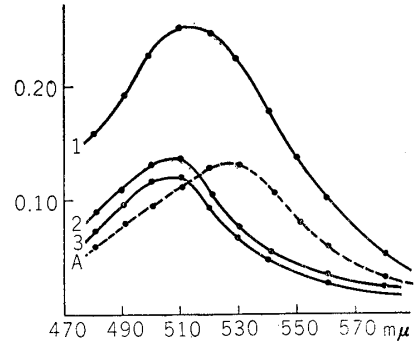

Fig. 4. Absorption Spectra of E. 1 in Elson-Morgan Reaction after Hypoiodite or Periodate Oxidation and Acid Hydrolysis

Curve 1 : without oxidation
〃 2 : hypoiodite oxidation
〃 3 : periodate oxidation
〃 A : glucosamine for control

Quantitative Periodate Oxidations of E. 1 and E′. 1—Table II represents the results of quantitative periodate oxidation of disaccharide E. 1 and its reduction product E′. 1. In this case, E. 1 slowly produced both formaldehyde and formic acid. It appears that the development of acidity was

Table II.

Quantitative Periodate Oxidation of Glucose, Acetylglucosamine, E. 1, and E′. 1

Substance	HIO_4 consumption or HCOOH, HCHO developed (mole)	0.1	1	2	5	9	17	24	48	156
Glucose	HIO_4 consumption	—	—	—	—	—	4.86	—	—	—
	HCOOH developed	—	—	—	—	—	—	3.54	—	4.40
N-Acetyl-glucosamine	HIO_4	—	—	2.75	3.70	—	4.93	—	—	—
	HCOOH	—	—	—	—	—	—	2.08	—	2.78
E. 1	HIO_4	0.15	—	0.80	—	1.55	—	2.25	2.45	—
	HCOOH	—	0.16	0.25	—	0.40	—	—	0.60	—
	HCHO	0.08	—	0.13	—	0.33	—	—	0.58	0.98
E′. 1	HIO_4	0.83	1.16	—	—	—	1.83	1.91	2.00	—
	HCOOH	0.00	0.08	—	—	—	0.13	—	0.16	—
	HCHO	—	0.80	0.92	—	—	1.04	—	—	—

caused by over-oxidation. Foster, *et al.*[10,12] reported that caution must be exercised in interpretation of the reaction of N-acetylated amino sugar derivatives with periodate. More reliable information on the position of the glycosidic linkage in E. 1 was obtained by periodate oxidation of its reduced derivative. Sodium borohydride readily reduced E. 1 to the corresponding di-N-acetyl-di-itol (E'. 1). This alcohol rapidly (1 hr.) consumed ca. 1 mole of periodate to release ca. 1 mole of formaldehyde with no liberation of formic acid in a short period.

Discussion

Structure of Disaccharide

According to the recent paper of Strange, *et al.*[5,13] the most probable structure for muramic acid is 3-O-(2-carboxyethyl)-hexosamine. From the evidences described above, the disaccharide is composed of N-acetylglucosamine and N-acetylmuramic acid, and the reducing group of the latter is concerned with its glycosidic linkage. Thus, no consumption of periodate should occur for acetylmuramic acid portion and structure of the disaccharide, E. 1, should be represented by (I), (II), or (III) in Chart 1.

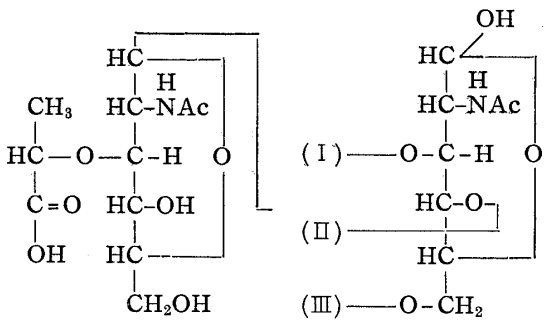

Chart 1.

In these structures, (III) (1→6) is precluded since the release of formaldehyde was observed with both E. 1 and E'. 1 on periodate oxidation. Between the two structures, (I) and (II), the latter is more conceivable because the Morgan-Elson coloration[14~16] of E. 1 is weak (ca. one-third of the color intensity produced by an equimolar quantity of acetylglucosamine) and E'. 1 releases 1 mole of formaldehyde but negligible amount of formic acid during the course of oxidation.

It is concluded that the disaccharide, E. 1, is the smallest fragment produced by the hydrolysis of muco-complex from *M. lysodeikticus* and it consists of 1 mole each of acetyl glucosamine and acetylmuramic acid, bonded by 1—4 linkage, as represented by the structure (II) in Chart 1.

Recently, Park, *et al.*[9] proposed a speculative structure of a disaccharide consisting of N-acetylglucosamine and N-acetylmuramic acid as a lysozyme-hydrolysis product of muco-complex from bacterial cell walls. His structural formula involves a linkage of the reducing group of N-acetylglucosamine to C-6 position of N-acetylmuramic acid. Thus, his proposal is comparable to the present result.

Constitution of Muco-complex

From the observation obtained this time, partial structure of substrate (muco-complex) and specificity of lysozyme should be represented as shown in Chart 2.

12) A. B. Foster, D. Horton : J. Chem. Soc., **1958**, 1890.
13) R. E. Strange, L. H. Kent : Biochem. J., **71**, 333(1959).
14) D. Aminoff, W. T. J. Morgan W. M. Watkins : *Ibid.*, **51**, 379(1952).
15) R. Kuhn, G. Krüger : Ber., **89**, 1473(1956).
16) J. A. Cifonelli, A. Dorfmat : J. Biol. Chem., **231**, 11(1958).

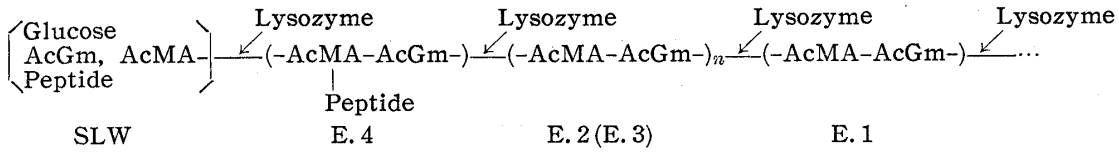

Chart 2. Supposed Partial Structure of the Substrate (Muco-complex) for Lysozyme

The substrate, muco-complex contains disaccharide units represented by (II) in Chart 1 and as for the linkage between these units in substrate muco-complex, 1-4 linkage of N-acetylglucosamine and N-acetylmuramic acid is the most probable from the report[17] that chitin is slowly split by lysozyme. Lysozyme should be specified as a kind of glucosaminidase.

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Summary

A purified lysozyme-hydrolysis product of muco-complex obtained from *M. lysodeikticus* was further investigated by hypoiodite and periodate oxidations as well as by electrometric titration to elucidate the structure of O-(N-acetylmuramic acid)-(1→4)-N-acetylglucosamine. The enzymatic specificity of lysozyme for a proposed structure of the muco-complex was discussed.

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[Added in proof] After this paper was submitted for publication, the paper of M. R. J. Salton and J. M. Ghuysen (Biochim. et Biophys. Acta, **36**, 552(1959)) and of H. R. Perkins (Biochem. J., **74**, 182(1960)) became available in this Library.

They proposed for the similar disaccharide with that treated in this paper a structure of 6-O-(N-acetylglucosaminyl)-N-acetylmuramic acid with some discrepancy from that reported by the author. The problem concerning the difference in these structures will be discussed in a forthcoming paper.

17) I. R. Berger, R. S. Weiser : Biochim. et Biophys. Acta, **26**, 517(1957).