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71. Otomatsu Hoshino : Studies on the Constitution of Muco-complex from *Micrococcus lysodeikticus*. IV.[1] Separation of Lysozyme-digested Products.

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In a previous paper[1] of this series, the results of studies on acid-hydrolysis products of the water-soluble muco-complex (fraction GII) obtained from bacterial cells of *Micrococcus lysodeikticus* was reported. A further purified fraction (GIIs) of fraction GII showed homogeneity on electrophoresis and was digested by lysozyme.[2]

Several reports dealing with the bacterial substrate of lysozyme obtained from *M. lysodeikticus* have appeared. Meyer, *et al.*,[3,4] and Epstein and Chain[5] reported a bacterial component hydrolyzed by the enzyme, giving reducing substances and an acetylhexosamine. Further, Salton[6] observed that water-insoluble cell walls from this bacteria were mainly composed of muco-complex active as substrate of the same enzyme.[7] However, the present series of work is the first one to achieve isolation of electrophoretically pure water-soluble muco-complex as the substrate of lysozyme from *M. lysodeikticus*.

As for the decomposition products of muco-complex of *M. lysodeikticus* by lysozyme, Schütte and Krisch[8] recently reported some results, but their result seemed to be somewhat complicated due to impurity of the starting material.

The present paper deals with the isolation of fraction GII, the pure muco-complex isolated in the preceding work on a large scale, as well as the fraction of its lysozyme hydrolysis products, including some aspects obtained on the structure of some of the products.

Experimental

Culture of Bacteria—30 L. of nutrient agar culture broth (beef extract 1%, yeast extract 0.1%, glucose 1%, peptone 1%, NaCl 0.2%, agar 3%) was warmed and 2 L. each of this medium was poured into culture plates made of stainless steel (35×50×10 cm.) After the bacteria was cultured at 37° for 48~72 hr., the organisms were treated by procedures outlined in a previous paper.[9] Yield of wet cells, 500~550 g.; dried cells, 80~100 g.

Separation of Fraction GII—Crude muco-complex was separated from dried bacteria by the modified method given in the first paper.[9] To 80 g. of acetone-dried bacteria 30 g. of KOH and 1 L. of water were added and this suspension was allowed to stand at 25° for 2 weeks. To this mixture, 5 cc. of AcOH and 10 g. of CCl_3COOH were added and diluted with water to 1.4 L. After removal of the precipitate formed on addition of equivolume of EtOH, equivolume of EtOH was added to the supernatant. The precipitate (about 11.8 g. in dry state) was dissolved in 100 cc. of water and the solution was shaken with 100 cc. of mixed solvent of $CHCl_3$—iso-AmOH (1:1). After centrifugation, the sediment was washed twice with water, the washings were combined with the supernatant (ca. 150 cc.), and the combined solution was subjected to EtOH fractionation. Precipitate formed between a concentration of 50~75% EtOH was washed with Me_2CO and Et_2O, and dried *in vacuo*.

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1) Part III. S. Akiya, O. Hoshino : This Bulletin, 8, 399(1960).
2) Part II. *Idem* : *Ibid.*, 8, 395(1960).
3) K. Meyer, J. W. Palmer, R. Thompson, D. Khorazo : J. Biol. Chem., **113**, 479(1936).
4) K. Meyer, E. Hahnel : *Ibid.*, **163**, 723(1946).
5) L. A. Epstein, E. Chain : Brit. J. Exptl. Pathol., **21**, 339(1940).
6) M. R. J. Salton : Biochim. et Biophys. Acta, **10**, 512(1953).
7) *Idem* : *Ibid.*, **22**, 495(1956).
8) E. Schütte, K. Krisch : Z. physiol. Chem., Hoppe-Seyler's, **311**, 121(1958).
9) Part I. S. Akiya, O. Hoshino : Yakugaku Zasshi, **77**, 777(1957).

Yield of crude muco-complex was 7~8 g.

The acrinol method described in the first paper[9] was applied to a solution of this crude muco-complex to obtain the fractions H and G. Fraction G was refractionated with EtOH as described in the second paper[2] to obtain fractions GI, GII, and GIII. Yield of GII was 4~5 g.

Degradation of GII with Lysozyme—To a solution of GII dissolved in 20 cc. of 0.05*N* acetate buffer (pH 5.5), 5 mg. of lysozyme*2 in 5 cc. of the same buffer solution was added and the mixture was incubated at 37° for 1 week.

(1) Viscosity: 1.5 cc. of the lysozyme-added solution of GII was immediately measured by the Ostwald viscosimeter for fall in viscosity at intervals, at 37°.

(2) Reducing Power: Liberation of reducing substances was estimated by the Somogyi's method with 0.2 cc. of the solution pipetted at intervals and diluted with water to 2 cc.

(3) Estimation of acetylamino-sugar: 0.2 cc. of the solution was taken at intervals, diluted to 1 cc., and increase in absorption intensity was measured at 510, 520, 530 mμ by the modified Morgan-Elson method.

Electrodialysis of Lysozyme Digest of GII—200 mg. of GII was digested with lysozyme as above and 200 cc. of the digest solution was electrodialysed against water at 200 v, 50 mA. After 24 hr. the current dropped to 5 mA. Dialysates in both cathode and anode chambers were evaporated *in vacuo*. Only inorganic substances were found in the dialysate from cathode chamber. From dialysate in anode chamber, a syrup was obtained which gave, on drying *in vacuo*, ca. 60 mg. of hygroscopic powder. After removal of the precipitate formed on addition of 3 volumes of EtOH to non-dialysable fraction, the supernatant was concentrated *in vacuo* and the concentrate was dried with Me_2CO and Et_2O to obtain 110 mg. of white powder.

Separation of Lysozyme Digest of GII—1) To a solution of GII (1 g.) in 10 cc. of 0.05*N* acetate buffer (pH 6.0) 5 mg. of lysozyme and 0.1 cc. of toluene were added and the solution was allowed to stand at 37°. After 48 hr., additional 5 mg. of lysozyme and after 72 hr. 1 cc. of Dowex-50 (H^+) were added. The reaction mixture was centrifuged, 2 volumes of EtOH was added to the supernatant, and again centrifuged. The supernatant was evaporated *in vacuo* and the residue was extracted three times with warm MeOH and the MeOH-insoluble fraction was dried *in vacuo* to obtain a white powder (SLW). To concentrated MeOH extract, a pale yellow syrup, Me_2CO and Et_2O were added and a hygroscopic white powder (SLM) was obtained on removal of the solvent *in vacuo*.

2) After digestion of GII (3 g.) with lysozyme as above, an equivolume of EtOH was added to the digest and the mixture was centrifuged. To the supernatant, 0.3 g. of $KHCO_3$ and 0.3 cc. of DNFB were added and shaken occasionally for 5 hr. EtOH was removed from the reaction mixture *in vacuo*, the residue was diluted with water, and extracted with Et_2O to remove unreacted DNFB. The aqueous layer was treated with a small volume of Dowex-50 (H^+) as described in (1). MeOH-insoluble pale yellow powder (SLDW) and MeOH-soluble yellow powder (SLDM) were obtained.

3) After digestion of DNP-GII (3 g.) with lysozyme, the digest was treated as (1) and MeOH-insoluble pale yellow powder (DSLW) and MeOH-soluble yellow powder (DSLM) were obtained.

4) After digestion of GII (1 g.) with lysozyme, the digest was treated with Dowex-50 (H^+) to remove lysozyme and adjusted with $NaHCO_3$ to pH 8.0. To the solution 5 mg. of trypsin*3 was added and incubated at 37°. After 24 hr., the trypsin-digest was dinitrophenylated with DNFB and the acidified solution was extracted successively with Et_2O and AcOEt. The aqueous layer was treated as described in (2). Each fraction and its hydrolysate were tested by paper electrophoresis and paper chromatography.

Charcoal-Celite Column Chromatography of MeOH-soluble Fraction—1) 10% solution of SLM (0.2 g. in 2 cc.) was fractionated through a charcoal-Celite column (2×25 cm.) made of 5 g. of Norit and 5 g. of Celite, with EtOH-gradient (0~50%, 50% EtOH+1~5% pyridine). Each eluate (20 cc.) was evaporated *in vacuo* and examined by combined paper electrophoresis and paper chromatography.

2) 0.7 g. of SLM or SLDM was dissolved in water (5 cc.) and fractionated on charcoal-Celite column (5×35 cm.) of 20 g. of Norit and 20 g. of Celite. Elutions were carried similarly as in the case of (1) and each eluate (100 cc.) was evaporated *in vacuo* and lyophilized.

3) Lysozyme digests of GII and each fraction in subsequent separation procedure were examined by two-dimensional paper electrophoresis, followed by paper chromatography. Conditions of paper electrophoresis were similar to those reported in Part III.[1] Solvent for paper chromatography used were BuOH-EtOH-H_2O (2:1:1) and those reported in Part III.[1]

Constituents of Digestive Product—Each fraction separated as above was hydrolysed and analysed for components in hydrolysate, similarly as reported in Part III.[1]

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*2 Lysozyme was prepared from egg-white by the bentonite adsorption method of Alderton, *et al.*[10] After crystallization the enzyme was dialysed and lyophilized.

*3 Trypsin was kindly supplied by the Mochida Pharmaceutical Company Laboratory.

10) G. Alderton, W. R. Ward, H. L. Fevold: J. Biol. Chem., **157**, 43(1945).

Results and Discussion

Changes in several physicochemical properties of fraction GII by the action of lysozyme, decrease in viscosity, and increase in both reducing power and Morgan–Elson's coloration, were plotted at intervals and the results are given in Figs. 1, 2, and 3, respectively. These results showed a rapid decrease of viscosity to reach a constant value (ca. 2 hr.) and a relatively slow increase of both reducing power and Morgan–Elson coloration to a constant value (ca. 24~48 hr.). GII was treated with lysozyme at its optimum pH (5.0~6.0) in acetate buffer according to earlier literature.[4,7]

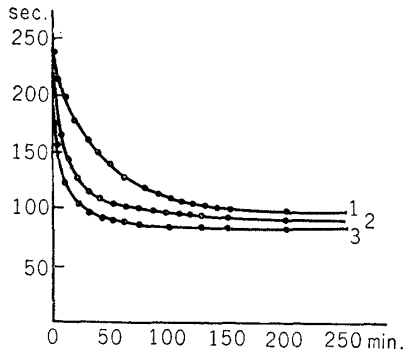


Fig. 1.
Viscosity Measurement 2% Buffered Solution of GII (GII : lysozyme = 100 : 1)

Buffer :
Curve 1 : 0.05*N* acetate (pH 5.5)
〃 2 : 0.1*N* acetate (pH 5.3)
〃 3 : 0.1*N* acetate (pH 5.0)

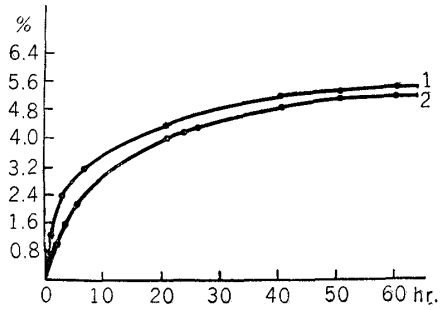


Fig. 2.
Development of Reducing Substances liberated by Lysozyme Action (expressed as glucose)

Buffer :
Curve 1 : 0.1*N* acetate (pH 5.0)
〃 2 : 0.05*N* acetate (pH 5.5)

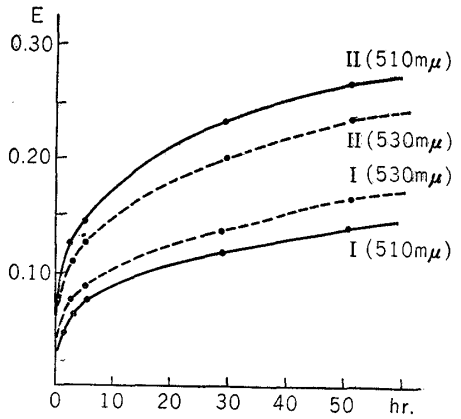


Fig. 3.
Estimation of Morgan–Elson Reactive Substances liberated by Lysozyme Action

I Measured after 3 hr.
II Measured after 24 hr. from color development

The hydrolysate was fractionated by electrodialysis. The dialysable product travelled toward the anode amounted to ca. ⅓ of the substrate used and was proved by paper chromatography to be a mixture of small molecular decomposition products.

For the treatment of a large amount of hydrolysate, a series of fractionation using ion-exchange resin, EtOH precipitation, and charcoal-Celite column chromatography was applied. In the course of fractionation shown in Chart 1, each fraction separated at

Hydrolysate of GII (substrate, lysozyme in acetate buffer)
↓ Dowex-50, centrifuged, and 2 vol. EtOH added
Precipitate (P. 1) | Supernatant
↓ evapd. *in vacuo*
Residue (SL)
↓ extd. with MeOH
MeOH-soluble Fraction (SLM) | Residue (SLW)
↓ Charcoal-Celite column chromatography | ↓ repptd. with Me_2CO, EtOH
↓ eluted with H_2O-EtOH mixture | SLW'
Eluates I～X
↓ evapd. and lyophilized
E.1 from Eluate V
E.2 from Eluate VI
E.3 from Eluate VIII
E.4 from Eluate IX

Chart 1.
Fractionation of Hydrolysis Products liberated by Lysozyme from Fraction GII

each step was examined for its purity by both paper electrophoresis and paper chromatogaphy. The result of such detection for the dried supernatant (SL) obtained by EtOH fractionation is given in Fig. 4 which shows five spots of hydrolysis product (SL-1～SL-5).

SL was extracted with MeOH to give MeOH-soluble (SLM) and -insoluble (SLW) portions. SLW mainly contained the product corresponding to spot SL-5 which did not move in paper chromatography. SLM mainly contained the spot SL-1 contaminated with a small amount of SL-2, SL-3, and SL-4. After further purification of SLM by charcoal-Celite column chromatography, only SL-1 eluted from the column with 20～30% ethanol.

SL-1 contained in acid eluate was unstable for vacuum concentration of the solution at above 50° and during the treatment, the gradually colored solution gave an evidence of partial decomposition of SL-1 into two compounds (SL-1A and SL-1B) which were detected on combined paper chromatogram by benzidine-trichloroacetic acid reagent (Fig. 5). From its Rf value on paper chromatogram, its mobility in paper electrophoresis, and its Morgan-Elson coloration, SL-1B was identified with acetylglucosamine. The mobility and Morgan-Elson coloration of SL-1A gave a probable identity of this compound with acetylmuramic acid.

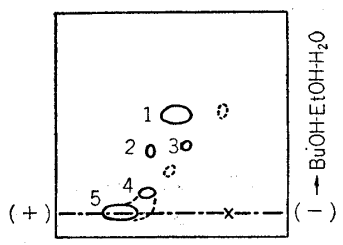


Fig. 4.
Two-dimensional Chromatogram of Fraction SL

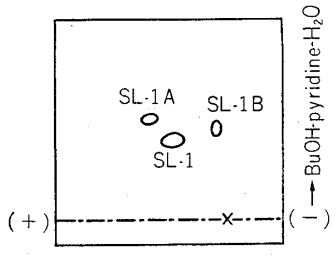


Fig. 5.
Two-dimensional Chromatogram of SL-1 and its Heat-decomposed Products (SL-1A, SL-1B)

With the detection of muramic acid in the acid hydrolysate of muco-complex, described in Part III of this series, the above results give identity of SL-1A as acetylmuramic acid. From these observations after decomposition of SL-1, it is evident that the latter is a labile disaccharide composed of acetylglucosamine and acetylmuramic acid. Fig. 6 shows the paper electrophoretic pattern of SL-1 during its decomposition in several intervals in heated aqueous solution.

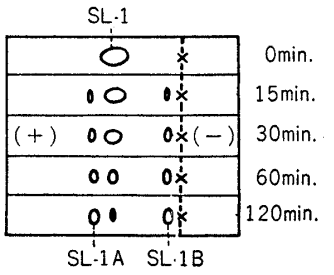
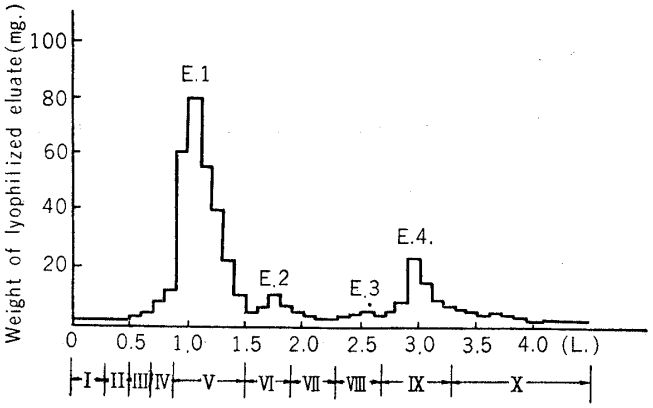


Fig. 6.
Paper Electrophoresis of SL-1, SL-1A, and SL-1B

The numbers expressed in min. show the intervals of heating the aqueous solution of SL-1.

Each of the eluates from charcoal-Celite column chromatography with 20~30% EtOH (E. 1), 40~50% EtOH (E. 2), 50% EtOH+1% pyridine (E. 3), and 50% EtOH+2% pyridine (E. 4) was concentrated to ⅓ the original volume at below 50° and lyophilized. Pure SL-1 was obtained from E.1, mainly SL-2 and SL-3 respectively from E.2 and E.3, and SL-4 from E.4. One example of above separations by charcoal-Celite column chromatography is illustrated in Fig. 7.



Solvent system :
I. Water
II. 5% EtOH
III. 10% EtOH
IV. 20% EtOH
V. 30% EtOH
VI. 40% EtOH
VII. 50% EtOH
VIII. 50% EtOH+1% pyridine
IX. 50% EtOH+2% pyridine
X. 50% EtOH+5% pyridine

Fig. 7. An Example of Charcoal-Celite Column Chromatography of Methanol-soluble Fraction (SLM) obtained from Lysozyme-digests of Fraction GII

Both SL-4 and SL-5 which gave faint tailing colorations with benzidine-trichloroacetic acid (Fig. 4) seemed to contain some contaminations. However, attempts to separate the main pure components of these fractions by cellulose column chromatography or zone electrophoresis were unsuccessful.

Yield of several fractions from the hydrolysate of GII with lysozyme is given in Table I.

Table I. Experimental Conditions of Lysozyme Digestion and Yield of Several Fractions in the Separation of Digest of GII

No.		Experimental Condition			Yield		
		Substrate (g.)	Lysozyme (mg.)	Trypsin (mg.)	P. 1 (mg.)	SLW (mg.)	SLM (mg.)
1	GII	1.0	10	—	200	300	250
2	GII	3.0	30	—	650	1,050	840
3	DNP-GII	3.0	30	—	700	1,100	800
4	GII	1.0	10	10	—	—	—

After reaction of 2,4-dinitro-1-fluorobenzene with GII and hydrolysis of DNP-GII with lysozyme, the hydrolysate was fractionated similarly as in the case of GII. In this case, besides the same spots for SL-1 and SL-2, two new yellow spots corresponding to those of compounds of dinitrophenylated SL-4 and SL-5 were observed.

After digestion of GII with lysozyme followed by removal of lysozyme, the digest was treated with trypsin to hydrolyse the peptide linkage concerned with the carboxyl group of lysine. The trypsin digest was treated with 2,4-dinitro-1-fluorobenzene and the resulting DNP-peptides were extracted with ether. The ether-soluble DNP-peptides were further fractionated by paper electrophoresis to obtain acidic DNP-peptides and the latter was further separated into two main fractions by paper chromatography. After acid hydrolysis and detection of componental amino acids of each acidic DNP-peptide eluted from cuttings of the filter paper, the peptides were proved to be DNP-Ala-Ala and DNP-Ala-(Ala-Gly). From above observations, it is assumed that the peptide portion of GII contains a linkage represented by -Lys-Ala-(Ala-Gly).

It is of interest that the proposed structure of UDP-N-acetyl-amino sugar peptide isolated by Park, *et al.*[11] from *Staphylococcus aureus* also contains a peptide part, the amino acid sequence of which is partially similar to that contained in fraction GII.

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Summary

A muco-complex (fraction GII) obtained from *Micrococcus lysodeikticus* in a preparative scale was hydrolyzed with lysozyme and the hydrolysate was fractionated into several components. From one of the fractions, a disaccharide consisting of acetylglucosamine and acetylmuramic acid was isolated. Further, a sequence of peptide linkage, -Lys-Ala-(Ala-Gly), in the muco-complex was established.

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