BIKEN JOURNAL
Preliminary Report

Soluble Lysozyme-Substrate Isolated from *Micrococcus lysodeikticus* Cell Walls by *Flavobacterium* L_{11} Enzyme Digestion

Immunochemical studies on lysozyme have been performed in one of the authors' laboratories for several years (Fujio *et al.*, 1959; Shinka *et al.*, 1962; Fujio *et al.*, 1962). In these studies, to elucidate the relation between the neutralizing and non-neutralizing antibodies in anti-lysozyme rabbit sera, a soluble lysozyme-substrate of small molecular size was required. It has recently been reported that an enzyme produced by a *Flavobacterium* species (tentatively named the L_{11} enzyme) exhibits a marked cell wall lytic activity towards *Micrococcus lysodeikticus* as well as *Staphylococcus aureus* (Kotani *et al.*, 1959b; Kato *et al.*, 1962). The L_{11} enzyme differs from egg white lysozyme and lysozyme-like enzymes in the way in which induces cell wall lysis, as illustrated by the fact that the lysis of *Staphylococcus aureus* cell walls by this enzyme is almost exclusively accompanied by an increase in free amino groups, but not by uncovering of reducing and hexosamine-reactive groups (Kato *et al.*, 1962). The experiments reported here were made to see if L_{11} enzyme digests of *Micrococcus lysodeikticus* cell walls could be used as substrate in the assay of lysozyme activity.

The cell wall preparations were isolated from cultures of *Micrococcus lysodeikticus* grown on nutrient agar supplemented with 0.5 per cent glucose and 0.1 per cent Bacto-yeast extract (Mayer and Hahnel, 1946) for 24 hours at 37°C, by the method previously described (Kotani *et al.*, 1959a; Kato *et al.*, 1962). The L_{11} enzyme was prepared as described by Kato *et al.* (1962). The method of preparation of the purified hen egg white lysozyme was reported by Fujio *et al.* (1959).

A specimen (1000 mg) of the cell wall preparations was suspended in 80 ml of 0.01 M phosphate buffer, pH 6.8, containing 200 units of the L_{11} enzyme (0.2 units enzyme/mg cell walls). The suspension was mixed with a few drops of chloroform as preservative and then incubated at 37°C for 48 hours. At the end of the incubation, the suspension, which was almost completely clear, was dialyzed three times in seamless cellulose tubing (Visking Co., U.S.A.) against 500 ml volumes of distilled water for 24 hours each. The non-dialyzable part of the digest, inside the tubing, was filtered through a sintered glass filter (3G4, Iwata Co., Osaka) and the filtrate (L_{11}-ND fraction, 120 ml containing 6.6 mg solids per ml) was used as the substrate for lysozyme assay. Recovery was found to be $6.6\ \text{mg} \times 120/1000\ \text{mg} = 79.2$ per cent.

The action of hen egg white lysozyme on the L_{11}-ND fraction was investigated as follows: Aliquots of 2 ml of the L_{11}-ND fraction were mixed with 1 ml aliquots of various concentrations of the lysozyme solution in test tubes which were then incubated in a water bath at 37°C for 60 minutes. Lysozyme was dissolved in

0.02 M phosphate buffer, pH 6.8, containing 0.01 per cent Merzonin (sodium ethylmercurithiosalicylate, Wako Pure Chemicals, Osaka). At the end of the reaction, 0.5 ml samples of the reaction mixtures were withdrawn from each of the tubes, and were assayed for their contents of N-acetylhexosamine by the method of Reissig, Strominger and Leloir (1955), which was modified by use of 0.1 M potassium tetraborate in place of 0.8 M sodium tetraborate. The determination of the N-acetylhexosamine content was also carried out on 0.2 ml aliquots of the reaction mixtures hydrolyzed in 0.125 N HCl, according to the description of Strominger (1957). In both determinations, the heating time in potassium tetraborate was prolonged from the 3 minute period given in the original papers to 50 minutes, since the extent of colour developed by test specimens was markedly increased by prolong heating without any appreciable increase in the colour exhibited by zero time controls. This fact suggests that not all of the split products released from the L_{11}-ND fraction under the action of lysozyme are free N-acetylhexosamines (*cf.* Ghuysen and Salton, 1960).

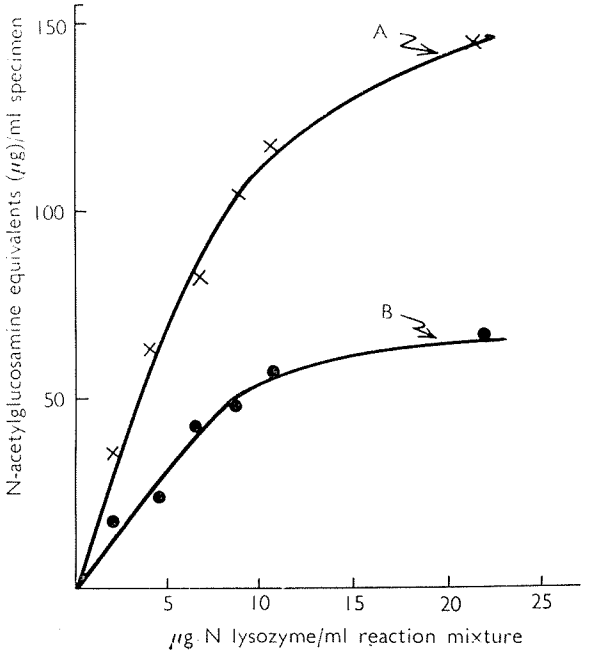

Fig. 1. Action of Hen Egg White Lysozyme on the Soluble Lysozyme-Substrate Isolated from *Micrococcus lysodeikticus* Cell Walls by Treatment with *Flavobacterium* L_{11} Enzyme

A: Determined on the specimen hydrolyzed in 0.125 N HCl according to the description of Strominger (1957).

B: Determined by the method of Reissig, Strominger and Leloir (1955) without the hydrolysis of specimens.

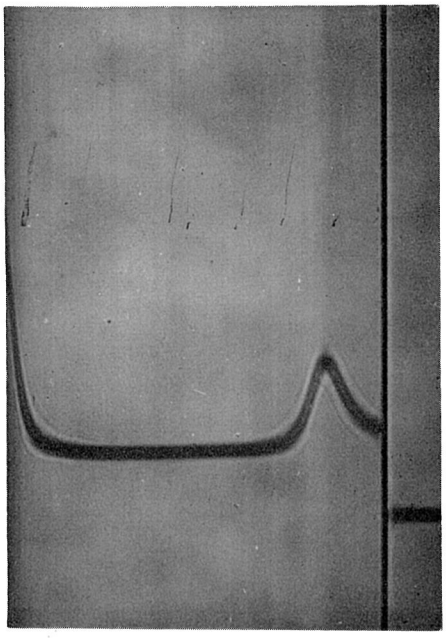

Fig. 2. Sedimentation Pattern of the Soluble Lysozyme-Substrate Isolated from *Micrococcus lysodeikticus* Cell Walls by *Flavobacterium* L11 Enzyme Treatment

Ultracentrifugation was performed in a Hitachi Ultracentrifuge (Model, UCA-1) at 17°C, on a specimen dissolved in 0.02 M phosphate buffer, 7.2, at a concentration of 1.2 per cent. The photogram was taken after centrifugation at 60,300 rpm for 111 minutes.

The results of the determinations are presented in Fig. 1. It can be seen that there is a linear relation between the amount (N-acetylhexosamine equivalents) of N-acetylhexosamine-reacting compounds released from the L_{11}-ND fraction by lysozyme and the amount of lysozyme added to the reaction mixtures when this is less than 7 μg N of lysozyme per ml. It is possible, therefore, to assay the lysoyme activity of test specimens using a standard curve.

The contents of ninhydrin-positive substances, hexosamines and hexoses in the L_{11}-ND fraction were estimated by the methods of Moore and Stein (1948), of Neuhaus and Letzring (1957) and by a modification of Scott and Melvin of the anthrone reaction (Colowick and Kaplan, 1957), respectively. The values of 63 per cent (as leucine), 24 per cent (as glucosamine-HCl) and 10 per cent (as glucose) were obtained. Fig. 2 illustrates the sedimentation pattern of the L_{11}-ND fraction, showing it as a single homogeneous component of $S_{17}=1.17$.

There have been several reports on soluble substrates for egg white lysozyme. Schütte and Krisch (1958) and Krisch (1958) reported the isolation of soluble lysozyme-substrate after treatment of *Micrococcus lysodeikticus* cells with 0.5 N NaOH

in an N_2-atmosphere, in experiments based on the work of Mayer and his colleagues (Mayer *et al.*, 1936; Mayer and Hahnel, 1946) and of Epstein and Chain (1940). Colobert and Dirheimer (1961) further purified the substrate isolated by the method of Schütte and Krisch by chromatography on a DEAE cellulose column. The simplest substrate hitherto reported is tetrasaccharide (AG-AMA-AG-AMA, AG: N-acetylglucosamine and AMA: N-acetylmuramic acid) isolated by Salton and Ghuysen from lysozyme-and *Streptomyces* F_1 enzyme-digests of *Micrococcus lysodeikticus* cell walls (Ghuysen, 1960; Salton and Ghuysen, 1959, 1960; Ghuysen and Salton, 1960). The soluble lysozyme-substrate reported here, however, has the distinct feature that it was isolated from *Micrococcus lysodeikticus* cell walls using *Flavobacterium* L_{11} enzyme and the lytic mechanism of this enzyme is basically different from those of egg white lysozyme and of F_1 enzyme. The further purification of the L_{11}-ND fraction and its chemical and physical properties are now being studied.

REFERENCES

Colobert, L. and Dirheimer, G. (1961). Action du lysozyme sur un substrat glycopeptidique isolé de *Micrococcus lysodeikticus*. *Biochim. Biophys. Acta* **54,** 455-468.

Colowick, S. P. and Kaplan, N. O. (1957). *Methods in Enzymology*, Vol. III, pp. 84-85, Academic Press, New York.

Epstein, J. M. and Chain, E. (1940). Some observations on the preparation and properties of the substrate of lysozyme. *Brit. J. Exptl. Pathol.* **21,** 339-355.

Fujio, H., Kishiguchi, S., Shinka, S., Saiki, Y. and Amano, T. (1959). Immunochemical studies on lysozyme. 1. Comparative studies of lysozyme and lysozyme methyl ester. *Biken's J.* **2,** 56-76.

Fujio, H., Saiki, Y., Imanishi, M., Shinka, S. and Amano, T. (1962). Immunochemical studies on lysozyme. III. Cross reactions of hen and duck lysozymes and their methyl esters. *Biken J.* **5,** 201-226.

Ghuysen, J. M. (1960). Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. II. Enzymic sensitivity of purified acetylhexosamine and acetylhexosamine-peptide complexes. *Biochim. Biophys. Acta* **40,** 473-480.

Ghuysen, J. M. and Salton, M. R. J. (1960). Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. I. Isolation and composition of acetylhexosamine and acetylhexosamine-peptide complexes. *Biochim. Biophys. Acta* **40,** 462-472.

Kato, K., Kotani, S., Matsubara, T., Kogami, J., Hashimoto, S., Chimori, M. and Kazekawa, I. (1962). Lysis of *Staphylococcus aureus* cell walls by a lytic enzyme purified from culture supernatants of *Flavobacterium* species. *Biken J.* **5,** 155-179.

Kotani, S., Hirano, T., Kitaura, T., Kato, K. and Matsubara, T. (1959a). Studies on the isolation of bacteria capable of lysing lysozyme-resistant, pathogenic bacteria. *Biken's J.* **2,** 143-150.

Kotani, S., Kato, K., Matsubara, T., Hirano, T. and Higashigawa, M. (1959b). *Staphylolytic* activity of a culture filtrate of a *Flavobacterium* species, isolated from soil. *Biken's J.* **2,** 211-213.

Krisch, K. (1958). Zur papierchromatographischen Bausteinanalyse des Lysozym-Substrates. *Hoppe Seyler's Z. Physiol. Chem.* **311,** 131-135.

Mayer, K., Palmer, J. W., Thompson, R. and Khorazo, D. (1936). On the mechanism of lysozyme action. *J. Biol. Chem.* **113,** 479-486.

Mayer, K. and Hahnel, E. (1946). Estimation of lysozyme by a viscosimetric method. *J. Biol. Chem.* **163,** 723-732.

Moore, S. and Stein, W. H. (1948). Photoelectric ninhydrin method for use in the chromatography

of amino acids. *J. Biol. Chem.* **176,** 367-388.
Neuhaus, O. W. and Letzring, M. (1957). Determination of hexosamines in conjunction with electrophoresis on starch. *Analyt. Chem.* **29,** 1230-1233.
Reissig, J. L., Strominger, J. L. and Leroir, L. F. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.* **217,** 959-966.
Salton, M. R. J. and Ghuysen, J. M. (1959). The structure of di- and tetrasaccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme and the $\beta(1\rightarrow4)$ N-acetylhexosaminidase activity of these enzymes. *Biochim. Biophys. Acta* **36,** 552-554.
Salton, M. R. J. and Ghuysen, J. M. (1960). Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. III. The structure of di- and tetra-saccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme. *Biochim. Biophys. Acta* **45,** 355-363.
Schütte, E. and Krisch, K. (1958). Über die Darstellung und Eigenschaften des sogenannten Lysozym-Substrates. *Hoppe Seyler's Z. Physiol. Chem.* **311,** 121-130.
Shinka, S., Imanishi, M., Kuwahara, O., Fujio, H. and Amano, T. (1962). Immunochemical studies on lysozyme. II. On the non-neutralizing antibodies. *Biken J.* **5,** 181-200.
Strominger, J. L. (1957). Microbial uridine-5'-pyrophosphate N-acetylamino sugar compounds. *J. Biol. Chem.* **224,** 509-523.

KEIJIRO KATO
SHOZO KOTANI
MICHIKO IMANISHI
TSUNEHISA AMANO

Department of Bacteriology
Nara Medical College
Kashiwara, Nara (K.K and S.K.)

Department of Immunology
Research Institute for Microbial Diseases
Osaka University (M.I. and T.A.)

Received on October 4, 1963