THE SPECIFICITY OF THE Vi-PHAGE II BACTERIOLYTIC ENZYME[x]

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Received August 7, 1970

Summary. Salmonella typhi infected with Vi-phage II produce an enzyme dissolving chloroform-killed, gram-negative bacteria. The enzyme was purified 200 times by chromatography on Amberlite IRC 50. The specificity of its action was investigated using Micrococcus lysodeikticus cells, S.typhi murein and muropeptide C3. It has been shown that the enzyme splits off the cross-linking peptide bond between the amino group of diaminopimelic acid and the carboxyl group of alanine.

During investigation of Vi-phages' develppment my interest concentrated on the enzyme abundantly produced by the Vi-phage II infected bacteria. Many phages provoke the synthesis of enzymes which dissolve chloroform-killed bacteria, for instance T2 /25/, T4 /24/, lambda /1/, N20F /16/. The specificity of muramidase has been established for some of them /13, 24, 25/; the mode of action of the others is still not elucidated /1, 16/. The purpose of the present study was to define the specificity of the Vi-phage II bacteriolytic enzyme.

Enzyme purification. For enzyme preparation the initial steps of Tsugita's /24/ procedure were adopted. Vi-phage II was grown on S.typhi phage type A, No.21802 in aerated mSB medium, as described earlier /23/. All subsequent operations were carried out at 4°. Clarified Vi-phage II lysate /2 l/ was centrifuged at 18000 g for 90 min. The phage sediment was removed and the supernatant was diluted with 4 l of water and 300 ml of 0.25% rivanol /6,9 diamino-2-etoxyacridine lactate/ were added. The solution was adjusted with acetic acid to pH 5.8 and left overnight. After centrifugation, the clear supernatant was passed

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[x]Partly supported by Polish Academy of Sciences

Abbreviations : DAP, diaminopimelic acid; FDNB, 1,2,4 fluorodinitrobenzene, DNP, 2,4 dinitrophenyl.

through Amberlite IRC 50 /100 mesh/ column 17 x 70 mm, equilibrated with 0.05 M phosphate buffer pH 5.8. The column was washed with 300 ml 0.1 M phosphate buffer pH 6.5 and phage enzyme was eluted with the same buffer, supplemented with NaCl to 0.6 M concentration. The active fractions were pooled, diluted with 4 volumes of water, acidified to pH 5.8 and applied to Amberlite IRC 50 /100 - 200 mesh/ 12 x 80 mm column. The column was washed with 0.1 M phosphate buffer pH .5 till the pH of the eluate reached the same value, then the enzyme was eluted with linear gradient concentration of NaCl from 0 to 0.6 M /100 ml 0.1 M phosphate buffer pH 6.5 + 100 ml 0.6 M NaCl in the same buffer/. The active fractions were pooled and concentrated in Visking dialysis tubing immersed in polyethylene glycol 6000. The enzyme could be kept in such solution at -10° for several weeks without any appreciable loss of activity, although it was not stable in other buffers.

This isolating procedure results in a 200 - 300-fold purification. The bacteriolytic activity of the purified enzyme per 1 mg protein was about ten times higher than that of the egg white lysozyme.

Preparation of substrates. M.lysodeikticus cells were prepared according to Litwack /10,11/. S.typhi murein was obtained as described previously /20/. The soluble substrate - muropeptide C3 /Fig.1/ was isolated from the lysozyme digests of S.typhi murein /21/, as described by Primosigh et al. /19/ for E.coli muropeptides, by preparative descending chromatography on MN 2114 FF paper, in the upper phase of n-butanol/acetic acid/water /4:1:5/, for 7 days. The substance was eluted, concentrated and purified further by electrophoresis on MN 2114 FF paper at 24 V/cm, 85 min., in pH 1.9 buffer composed of 14 ml formic acid, 30 ml acetic acid and water up to 1 l. The slowest moving, main fraction representing C3 was eluted and dried in a desiccator. The muropeptide C6 /Fig.1/ was also eluted from the chromatograms of lysozyme digests; it needed no further purification. Muropeptides C3 and C6 from S.typhi were indistinguishable from the corresponding muropeptides of E.coli /21/ by chromatography or electrophoresis.

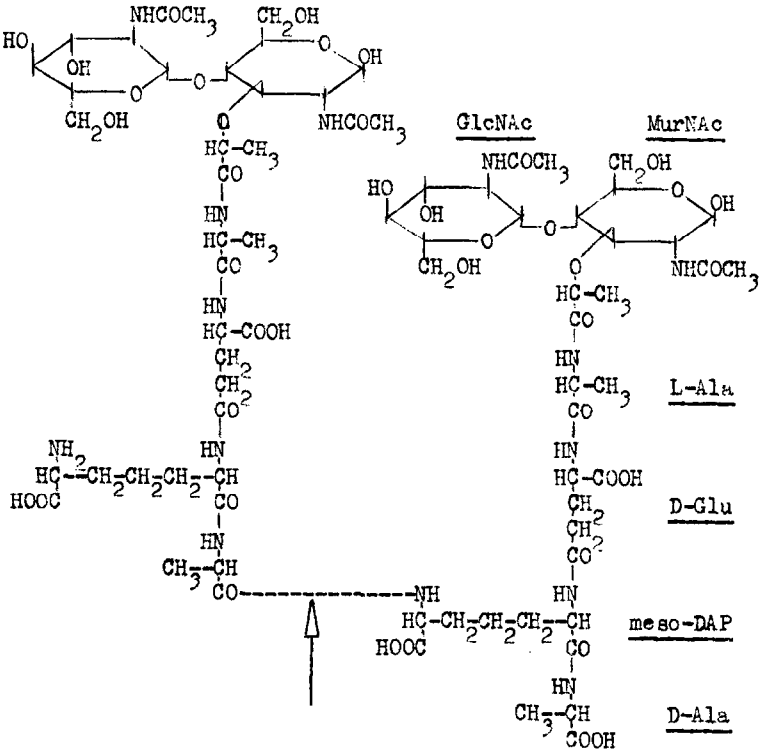

Fig.1. Muropeptide C3 : peptide-linked dimer of the two C6 units /14/. The arrow shows the connecting link.

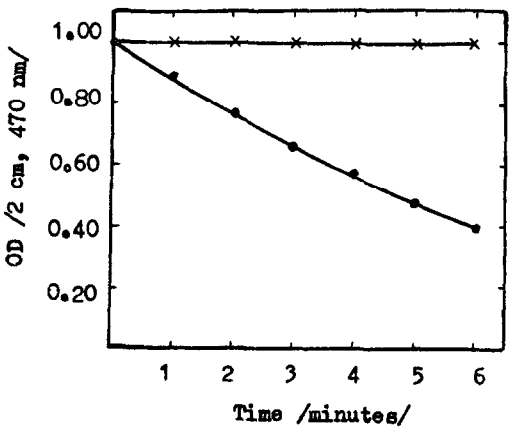

Fig.2. Reaction with M.lysodeikticus cells. M.lysodeikticus cells were suspended in 0.06 M phosphate buffer pH 6.2 at concentration 250 ug/ml, and optical density /2 cm, 470 nm/ was adjusted to 1.00. 100 µl of the phage enzyme solution /10 units/ml/ was added to 1.2 ml of the suspension and optical density was read at 1 minute intervals /x/.

Lysozyme /10 µg/ml/ activity was measured in the same conditions as a control /o/.

Reaction with M.lysodeikticus cells . At the beginning it was assumed that the phage enzyme may have the same specificity as the lysozyme, but it appeared /Fig.2/ that it does not affect M.lysodeikticus cells. Hence it differs in its specificity from lysozyme.

Reaction with S.typhi murein. The enzyme was dialysed against 200 volumes of 0.1 M ammonium acetate pH 6.5 for 17 hours at 4°. Murein, freeze-dried /52 mg/, was suspended in 20 ml 0.1 M ammonium acetate and 1.6 ml of enzyme solution /500 µg of protein, 5000 units/ was added. The digestion was carried out at 37°, in dialysis tubing /Visking/ immersed in 80 ml of 0.1 M ammonium acetate, with vigorous stirring. After 20 hours, 0.4 ml of the fresh dialysed enzyme solution /100 µg protein, 1000 units/ was added. At fixed time intervals samples were taken from dialysate for the determination of reducing groups, free amino groups and dry weight of dialysable products, according to Ghuysen /6/. Dry weight was calculated in µM assuming that 1 murein subunit, corresponding to C6 muropeptide, has a molecular weight of 936. The results calculated in µM

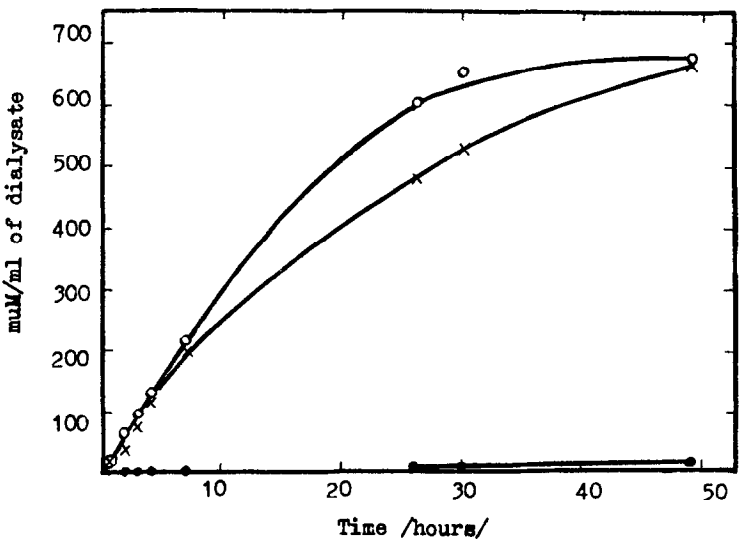

Fig.3. Phage enzyme action on S.typhimurein. The phage enzyme was incubated with S.typhi murein in dialysis tubing. Dry weight /o/, reducing groups /•/ and free amino groups /x/ were determined in the dialysate, according to Ghuysen /6/. For further details see the text.

per 1 ml of the dialysate are presented in Fig.3. The experiment does not reflect the reaction kinetics, since it is obscured by the uncontrolled velocity of dialysis.

It follows from the experiment that no reducing groups were liberated, while the amount of amino groups set free corresponded approximately to 1 muM per 1 muM of muropeptide C6. Hence the Vi-phage II bacteriolytic enzyme appeared to be a peptidase or amidase.

Reaction with muropeptide C3. The muropeptide C3 was chosen as a soluble substrate because its structure is well known /3, 7, 14/ and it is the simplest murein structural unit which contains all kinds of peptide bonds present in murein, together with the amide bond between muramic acid and alanine /Fig.1/.

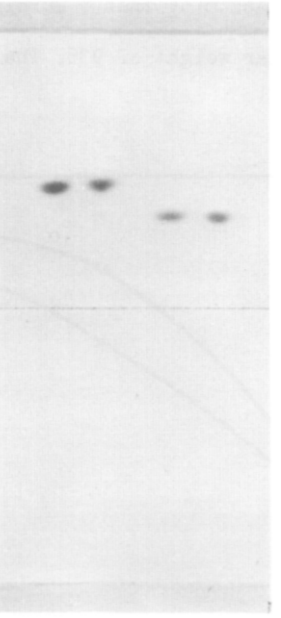

Fig.4. Electrophoresis of muropeptide C3 digested by phage enzyme. Electrophoresis was performed on cellulose MN 300 plates 9 x 20 cm, at 20 V/cm, for 85 min., in formic/acetic acid buffer pH 1.9. 1 µl samples were applied in the central line of the plate. No.1 - muropeptide C6. No.2 - product of C3 digestion by the Vi-phage II bacteriolytic enzyme. No.3 - muropeptide C3 after lysozyme treatment /unchanged/. No.4 - muropeptide C3. The spots were revealed with 0.2% ninhydrin in acetone.

Muropeptide C3 /400 µg/ was subjected to digestion by phage enzyme /20 µg of protein, 200 units/ in 200 µl of 0.1 M ammonium acetate pH 6.5, with a drop of toluene, at 37°, overnight. A parallel digestion with 200 µg of lysozyme was carried out in the same conditions. Most of the ammonium acetate was removed by sublimation in vacuum, over NaOH pellets. The products of the reaction were dissolved each in 40 µl of 10% isopropanol in water, and examined by thin layer electrophoresis and chromatography. The electrophoresis pattern is shown on Fig.4. In electrophoresis the product of digestion by phage enzyme resembled C6 and proved to be identical with C6 on thin layer chromatograms developed with n-butanol/acetic acid/water /4:1:5/ and n-amyl alcohol/isobutanol/n-propanol/pyridine/water /5:5:5:15:15/ /4/. These results strongly suggest

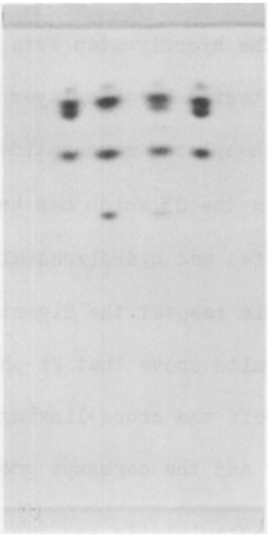

Fig.5. Electrophoretic pattern after dinitrophenylation and hydrolysis of muropeptide C3 digested by phage enzyme. 1 µl samples were applied to MN 300 cellulose plates 9 x 20 cm and subjected to electrophoresis at 22 V/cm, for 45 min., in formic/acetic acid buffer pH 1.9. No.1 and 4 - reference substances, from top to bottom : glucosamine, alanine, DAP, muramic acid + glutamic acid. No.2 - muropeptide C3 digested by Vi-phage II bacteriolytic enzyme, dinitrophenylated and hydrolysed; all DAP is converted into mono-DNP-DAP. No.3 - muropeptide C3 dinitrophenylated and hydrolysed; both DAP and mono-DNP-DAP are present. The spots were revealed with 0.2% ninhydrin in acetone.

that the phage enzyme splits off the peptide linkage joining two C6 units in their dimer - muropeptide C3, namely, the linkage between the amino group of DAP and the carboxyl group of alanine.

To confirm this suggestion, dinitrophenylation of C3 and of the enzyme-digested C3 was carried out, according to Ghuysen /6/. In C3 only one of two DAP residues has an amino group accessible to dinitrophenylation, the other one being engaged in the cross-linking peptide bond with alanine /Fig.1/. In C6 each DAP residue is converted into mono-DNP-DAP by dinitrophenylation and hydrolysis. Muropeptide C3 /200 µg/ and the phage enzyme-digested C3 /200 µg/, dried on the bottom of test tubes, were dissolved each in 100 µl of 1% $K_2B_4O_7$ and treated with 10 µl of FDNB reagent /130 µl of FDNB in 10 ml of ethanol/. The solutions were heated 30 min. at 60° and extracted 3 times with ethyl ether. The water phases were dried and hydrolysed in 4 N HCl, 17 hours at 103°. HCl was removed in vacuum, over NaOH. Standard mono-DNP-DAP was prepared according to Mirelman and Sharon /15/. The hydrolysates were dissolved each in 50 µl of 10% isopropanol in water, and tested by thin layer electrophoresis /Fig.5/. In the hydrolysate of dinitrophenylated muropeptide C3 both DAP and mono-DNP-DAP are present /Fig.5/, whereas in the C3 which has been digested by Vi-phage II enzyme and then dinitrophenylated and hydrolysed all DAP is converted into its mono-DNP-DAP derivative. In this respect the digested C3 is again identical with muropeptide C6. These results prove that Vi-phage II bacteriolytic enzyme is an endopeptidase splitting off the cross-linking bond in the S.typhi murein between the amino group of DAP and the carboxyl group of alanine.

Discussion. The synthesis of bacteriolytic enzymes by phage-infected bacteria is often observed /1, 16, 24, 25/, but little is known about the specificity of their action. The enzyme determined by gene e of the phage T4 is a muramidase /24/, resembling the lysozyme of the egg white. The enzyme determined by gene R of the phage lambda is called lysozyme, but its specificity has not been elucidated; moreover, it does not act on M.lysodeikticus nor on the products of li-

Table 1. Purification of phage enzyme

	Total activity units	Recovery %	Specific activity units/ug protein	Purification factor
Supernatant 18000 g	16000	100	0.05	1
Rivanol treatment	18000	110	0.03	1
First IRC 50 column	5800	36	0.34	11
Second IRC 50 column	5000	31	10.00	200

Table 1. The bacteriolytic activity was measured by following the decrease of turbidity of the bacterial suspension, 1 unit corresponding to the activity of 1 ug of egg white lysozyme /Sigma, 3 x cryst./. Freeze-dried E.coli B cells grown on M9 medium and prepared according to Tsugita et al./24/ served as a substrate. Suspension of E.coli cells in 0.05 M tris buffer pH 7.4 was adjusted to optical density 1.6 /2 cm, 470 nm/. 1 ml of the suspension was mixed with 100 ul enzyme solution, incubated exactly 4 min. in water bath at 37°, and immediately cooled. The turbidity was read in 2 cm cuvettes at 470 nm against bacterial suspension with 100 ul of buffer added. Standard curve was prepared with 1 - 7 ug/ml solutions of egg white lysozyme.

Protein was determined by the method of Lowry et al. /12/. Bovine serum albumine was used as standard.

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mited hydrolysis of chitin /1/. In this context it is interesting to note, that the Vi-phage II bacteriolytic enzyme, resembling the lambda "lysozyme", appeared to be an endopeptidase hydrolysing the cross-link between the amino group of DAP and the carboxyl group of alanine.

Several enzymes of this specificity were isolated from microorganisms : KM endopeptidase /5/ and partially purified L3 enzyme /9/ from Streptomyces, and mucoendopeptidase /17, 18/ from autolytic E.coli B system, which appeared to be identical /2/ with carboxypeptidase I /8/ from E.coli B.

The Vi-phage II endopeptidase may be engaged in the final lysis of the phage-infected bacteria. It is not known if it cooperates with the Vi-polysaccharide deacetylating enzyme of the Vi-phage II /22/ during the first steps of penetration.

Acknowledgements. The author wishes to thank Dr.R.Skórko for preparing the ionite, and Mrs.J.Żabina and Mrs.G.Bartnicka for their excellent technical assistance.

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