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BACTERIOLYTIC ENZYMES OF Vi PHAGE III LYSATE

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SUMMARY

Vi phage III infected Salmonella typhi cells were shown to contain two activities which lyse the chloroform-killed E. coli B cells. These enzymes have been separated by chromatography on CM-cellulose column and identified as the D-alanyl-meso-DAP endopeptidase and the N-acetylmuramyl-L-alanine amidase. The substrate specificity of these enzymes was investigated using low molecular weight muropeptides C3 and C6. It has been shown that muropeptide C3, the cross-linking unit in E coli B murein is completely resistant to the amidase action. This property of Vi phage III amidase suggested that this enzyme does not possess the ability to cause lysis, at the end of the production cycle, of host-bacteria infected with this phage.

The partially purified / 200-fold / bacteriolytic activity of Vi phage III infected Salmonella typhi lysate corresponds to the occurrence of N-acetylmuramyl-L-alanine amidase / 1 / which splits the amide bond between L-alanine and N-acetylmuramic acid in E. coli B murein. The release of amino groups during E. coli B murein digestion was not accompanied by the appearence of either hexosamines or reducing groups. The dialysable products of the murein and muropeptide C6 digestion were identical with tetrapeptide L-Ala - D-Glu - meso-DAP - - D-Ala.

Octapeptide L-Ala - D-Glu - meso-DAP - D-Ala
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L-Ala - D-Glu - meso-DAP - D- Ala, corresponding to the amidase action on the cross-linking fragments in E. coli B murein / 2-4 /, was not detected. However, the

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digestion products appeared to be about 70% w/w diffusible / 1 /. Hence, I suspected that this Vi phage III enzyme preparation would contain an unconfirmed enzyme splitting the cross-linking bond between the peptide subunits in E. coli B murein,

MATERIALS AND METHODS

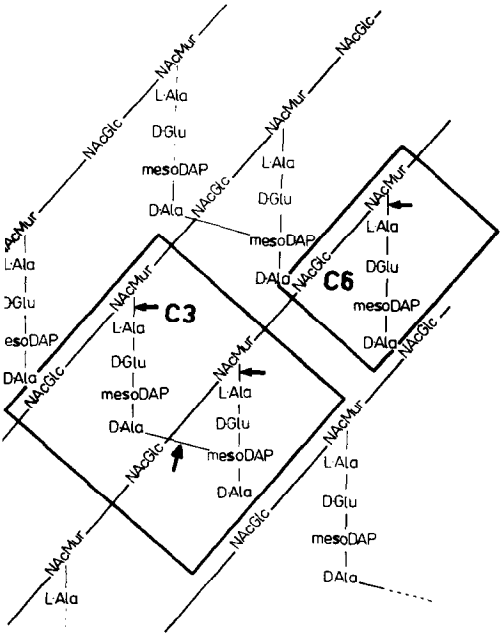
Most of the methods and materials used in this experiment were the same as those described previously / 1 /.

Bacteria and phages. Salmonella typhi 21802 phage type A and Vi phage III from the National Centre of Salmonella in Gdańsk, Poland, were used.

Enzyme preparation. Partially / 200-fold / purified bacteriolytic activity from Vi phage III lysate was obtained as described previously / 1 /.

Lytic activity assay. The medium and procedure for bacteriolytic activity assay were the same as those described previously / 1 /. One bacteriolytic activity unit / BAU / corresponding to the activity of 100 ng of egg white lysozyme / Sigma /.

Analytical methods. The reducing power was determined as described by Ghuysen et al. / 5 /, using N-acetylglucosamine / NAcGlc / as a standard. Protein was determined by the method of Lowry et al. / 6 /, using egg white lysozyme as a standard. Free amino groups were determined by a modified method of Obata et al. / 7 /. The dried sample was diluted in 3 ml of 0.5 M phosphate buffer pH 7.5 and 1 ml of 16 mM 2,4,6-trinitrobenzenesulphonic acid / TNBS / in methanol. added. After 1 h incubation at room temperature in the dark, the $E^{1\ cm}_{422\ nm}$ was measured. The standard curve was prepared with alanine.



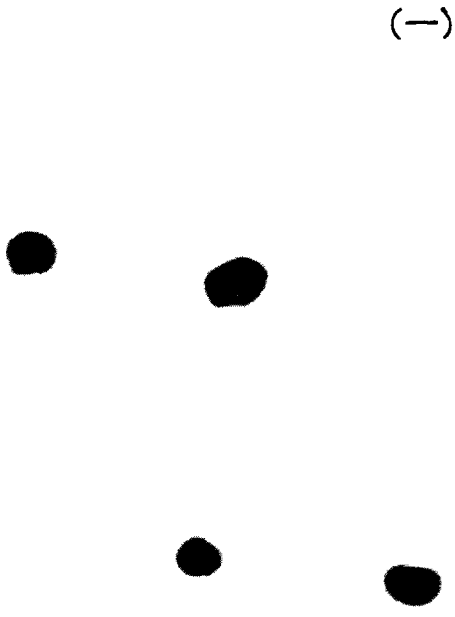


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Fig. 1. The schematic representation of the fragment of E. coli B murein with the muropeptide C3 and C6. The arrows show the amidase ◀— and endopeptidase ◁— action.

Fig. 2. Electrophoresis of muropeptide C3 digested by partially purified Vi phage III enzyme. The electrophoresis was performed on MN300 cellulose plates in 2% formic acid pH 2.0 at 18 V/cm for 1 h. From left to right: tetrapeptide; products of muropeptide C3 digestion; and muropeptide C3.

RESULTS

Reaction of 200-fold purified Vi phage III bacteriolytic enzyme with muropeptide C3. For investigating the possibility of the existence of two activities in the partially purified Vi phage III enzyme preparation, muropeptide C3 / 8 / was choosen as a soluble substrate / Fig. 1 /.

100 µg of muropeptide C3 in 0.5 ml of 1 mM ammonium acetate pH 7.4 was digested by a 200-fold purified enzyme preparation / 29 BAU /, with a drop of toluene, for 15 h at 37°C.

The resultant products were freed from ammonium acetate by sublimation in vacuo over NaOH pellets and examined by electrophoresis and thin layer chromatography / Fig. 2 /.

The results showed that only tetrapeptide L-Ala - D-Glu - - meso-DAP - D-Ala was obtained as product of the muropeptide C3 digestion. The expected product of amidase action with muropeptide C3, octapeptide L-Ala - D-Glu - meso-DAP - D-Ala
L-Ala - D-Glu - meso-DAP - D- Ala, was not detected. Thus, the 200-fold purified Vi phage III enzyme must contain amidase and another enzyme which cleaves the peptide cross-linking bond between two peptide subunits in muropeptide C3.

Separation of the two Vi phage III enzyme activities. For the separation of the enzymes 170 ml of 200-fold purified enzyme was applied to a CM-cellulose column / 150 x 15 mm / equilibrated with 50 mM Tris-HCl buffer pH 7.1. The column was washed with 1.5 l of 10 mM Tris-HCl buffer pH 7.1; no bacteriolytic activity in the eluate was detected. Subsequently, 10 mM Tris-HCl buffer pH 7.4 followed by a linear gradient of NaCl / 0 to 0.5 M / in the same buffer / total volume 300 ml / was used.

As can be seen from the elution profile / Fig. 3 / one of the bacteriolytic activities / fraction 1 / could be eluted by 10 mM Tris-HCl buffer pH 7.4 - the second / fraction 2 / by increasing the ionic strength of the same buffer.

Specificity of fractions 1 and 2 from CM-cellulose column. For comparision of these two bacteriolytic fractions, the amount of the amino groups and reducing power of the dialysable products of the E. coli B murein digestion were measu-

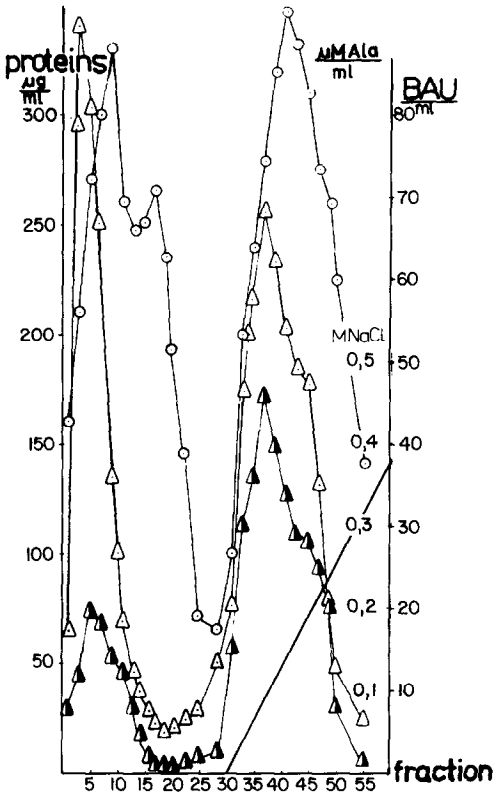

Fig. 3. Separation of the partially purified Vi phage III enzyme on a CM cellulose column. Fractions 2 - 10 - first bacteriolytic activity / fraction 1 /, and fractions 30 - 47 - second enzyme / fraction 2 /. —— molarity of NaCl, —△— bacteriolytic activity / BAU /, —○— proteins / ug/ml /, and —▲— "amidase" activity as the amount of free amino groups / uM Ala/ml / released from muropeptide C6 digestion, determined by the modified method of Obata / 7 /.

red. To 3.1 mg of lyophylized E. coli B murein, suspended in 1.5 ml of 10 mM Tris-HCl buffer pH 7.4, 200 BAU of each enzyme dialysed against this same buffer, and 3 drops of toluene were added. The blank samples were prepared in the same way, but the enzymes added were denaturared by 30 min. heating at 100°C. After 15 h incubation at 37°C the reaction was stopped by cooling in an ice bath, and the supernatant / 20000 x g for 1 h at 0°C / dried in vacuo over NaOH pellets. The dialysable

TABLE 1

Comparision of the enzymes of fractions 1 and 2 from the CM-cellulose column.

	Enzyme /BAU/mg/	The murein digestion products: free amino groups /nM Ala/	reducing power /nM NAcGlc/
Fraction 1	645	489.5	13.8
Fraction 2	1418	1741.1	16.1

For details see the text.

products of the reaction were used for free amino groups / 7 / and reducing power / 5 / assays / Table 1 /.

The results showed that these two bacteriolytic fractions strongly differ in their ability to release the free amino groups in reaction with E. coli B murein. According to these data fraction 1 contains about 30% of amidase activity from fraction 2. This is in agreement with Fig. 3 / amino groups released from monomer C6 /.

The specificity of these two bacteriolytic fractions for low molecular weight substrates was identified by examining the products of the digestion of muropeptides C3 and C6. To 100 µg of muropeptide dissolved in 0.5 ml of 1 mM ammonium acetate pH 7.4, 50 ul of enzyme solution / 30 BAU / dialysed against 1 mM ammonium acetate pH 7.4, and a drop of toluene were added. After incubation for 15 h at 37°C the resultant products were dried in vacuo over NaOH pellets and examined

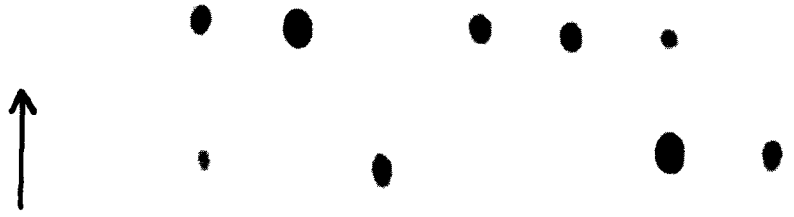

Fig. 4. Thin-layer chromatography of the digestion products of muropeptides C3 and C6 with the enzymes of fractions 1 and 2 from the CM-cellulose column. MN300 cellulose plates and Gerday's solvent system / 9 / were used. From left to right: muropeptide C3; muropeptide C3 digested by the enzyme of fraction 1; muropeptide C6; muropeptide C3 digested by the enzyme of fraction 2; muropeptide C6; muropeptide C6 digested by the enzyme of fraction 1 ; muropeptide C6 digested by the enzyme of fraction 2; tetrapeptide.

by thin layer electrophoresis in 2% formic acid / pH 2 / and chromatography in the solvent systems: 1. n-butanol - acetic acid - water / 4 : 1 : 5 by vol. upper phase /, and 2. n-amyl-alcohol - isobutanol - n-propanol - pyridine - water / 5 : 5 : 5 : 15 : 15 by vol. / 9 /, on MN300 cellulose.

Both the electrophoresis and chromatography / Fig. 4 / showed that fraction 1 contained an activity possessing the ability to liberate muropeptide C6 as a main product from muropeptide C3. This result proved that fraction 1 contained chiefly the D-alanyl - meso-DAP endopeptidase which splits the cross-linking bond between two peptide subunits in muropeptide C3, thus in murein. The action of the amidase contamination in this fraction was not detected in these tests. On the contrary, fraction 2 was not effective in the reaction with muropeptide C3, but muropeptide C6 digestion was accompa-

nied by the appearence of tetrapeptide L-Ala - D-Glu - meso-DAP - D-Ala. Thus this fraction contained the previously described N-acetylmuramyl-L-alanine amidase / 1 /, which cleaves only the amide bond between N-acetylmuramic acid and L-alanine in non-cross-linking peptide subunits in muropeptide C6 and in murein.

DISCUSSION

The results of this investigation show that the Vi phage III lysate, like the lysates of several phages / 10, 11, 2, 12-14 / contained two activities which digested the host-cell wall. The bacteriolytic activity of Vi phage III contains the N-acetylmuramyl-L-alanine amidase / 1 / and the second enzyme - D-alanyl - meso-DAP endopeptidase, which cleaves the peptide cross-linking in E. coli B murein / cf. 10, 11 /.

The occurrence of amidase in the lysates of several phages has been already reported /1, 15-18 /, but the physiological role of this enzyme in the phage production cycle is still unknown.

The data obtained in this investigation favors the supposition that only the endopeptidase activity is essential for host-cell lysis at the end of the latent period in the course of the phage development. The amidase which is <u>per se</u> a bacteriolytic enzyme / cf. 15-18 / is not an essential factor in the lysis of host-cells in vivo / cf. 19 /, because peptide units / see specificity for muropeptide C3 and C6 /, does not degrade the murein structure and its integrity.

The general condition of lysis / 19 / is the degradation of the murein structure / 20 /. Of the two enzymes studied

only endopeptidase can cause it because it breaks the cross-linkages.

It appears also that the rapid lysis of Vi phage III -infected S. typhi, obserwed at the end of the latent period, is probably the consequence of a joint action of both enzymes, endopeptidase preparing the substrate for amidase action.

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