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ACETYLATED PECTIC ACID AS A SUBSTRATE OF Vi PHAGES DEACETYLASE

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SUMMARY

The conditions of obtaining [^{14}C] acetylated pectic acid with a high specific activity are presented. On the basis of radioisotope measurements of the liberated, labelled acetic acid, the activity of deacetylase associated with the ViI, ViII and ViIII phage particle was determined. It has been shown that acetylated pectic acid is a substrate for the enzyme of those phages. The conditions of identifying the deacetylase are presented; it has been shown that the activity of Vi phage II enzyme is repressed by the phosphate buffer. In a system analogous to the one used for the Vi-polysaccharide, the recepter activity of the acetylated pectic acid can not be shown.

Vi polysaccharide / N,O acetylated / 1 - 4 /-2 amino--2 deoxy-α-D-galacturonan / isolated from bacteria is deacetylated by Vi phages / 1,2,3 /. The enzyme, deacetylase, is located at the end of its tail / 1,4,5 /. The deacetylase of Vi phage III / acetyl-α-1,4-galacturonan acylhydrolase / shows a similar substrate specificity towards Vi polysaccharide as to a modified substrate - O acetylated / 1 - 4 /-α--D-polygalacturonan / 6 /. The specificity of this enzyme of the morphologically different Vi phages / 7,8 / to this artificial substrate has not been tested.

In previous papers / 5,6,9 / acetic acid liberated in enzyme reaction, was identified by the gas-liquid-chromatography method. In the present study the method for [^{14}C] acetylation of pectic acid and a method for assaying the liberated acetic acid, based on radioactivity measurements, are described. This method makes possible a very quick de-

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termination of enzyme activity in different buffers, without introducing the inner standard and without determining the coefficients.

MATERIALS AND METHODS

Vi phages I, II and III were obtained from the International Reference Laboratory for Enteric Phage Typing in London. Vi phages I and II were propagated, as described previously / 1,3 /, on Salmonella typhi 21802 from the National Reference Laboratory for Enteric Phage Typing in Gdańsk, Poland. Vi phage III was propagated on Citrobacter Ci 23, as described elsewhere / 5 /. Lysates of Vi phage I and II were purified on ECTEOLA column and by differential centrifugation / 3 /. The lysates of Vi phage III, after being concentrated by the subpressure dialyse method, were centrifuged in density gradient of cessium chloride / 5 /. The phage titrations were carried out by Adams technique / 10 / using a S. typhi.

Isolation of Vi polysaccharide. Vi polysaccharide was isolated from an agar Citrobacter Ci 23 culture and purified on DEAE Sephadex A-25 column / 6 /.

Acetylation of pectic acid. The acetylation procedure with [^{14}C] anhydride of acetic acid was based on the method described elsewhere / 6 /. 500 mg of pectic acid /m.w.20000/ was dissolved in 20 ml of boiling formamide and then cooled to room temperature. Mixing this solution all the time, an equal volume of pyridine was added by drops / at the speed of 0.5 ml/min./. Subsequently, to this gel solution 5 ml [^{14}C] acetic acid anhydrite / 0.4 mCi/ml, obtained from IZOTOP - USSR, 12.4 mCi/mM and 98% of radiochemical purity / was added by drops / 0.5 ml/min./. The mixing of the solution was continued at room temperature for 3 hours. Next, 15 ml of the anhydrite, not labelled with the radioisotope, was added to it. After a night incubation at 27°C the solution was purified with ice, dialysed against a flowing water and lyophylized. The contaminated product was then purified on a DEAE Sephadex A-25 column / 2 x 25 cm, the speed of flowing 0.25 ml/min./, equilibrated with 50 mM Tris-HCl buffer pH 7.4. The adsorbed product was eluted from the column with a linear concentration gradient of NaCl / 0 - 0.8 M / in the same buffer. The concentration of acetylated pectic acid in each one of 2.5 ml fractions was determined by Webster et al. / 11 / method. The radioactivity of the fractions was measured with a grainend counter. The fractions eluted with 0.5 - 0.6 M of NaCl showed a high concentration of acetylated pectic acid and a high radioactivity / Fig. 1 /. Those fractions were pooled, dialysed against water and lyophylized. 350 mg of substrate, with a specific activity of 230 nCi/mg was obtained. The final product had one [^{14}C] acetyl group/1000. The degree of acetylation, determined by Snyder and Stephens / 12 / method, attained 18%

The radioactivity measurements of [^{14}C] acetic acid were carried out by means of a Nuclear Chicago scintillation counter, using the Bray scintillator system / 13 /.

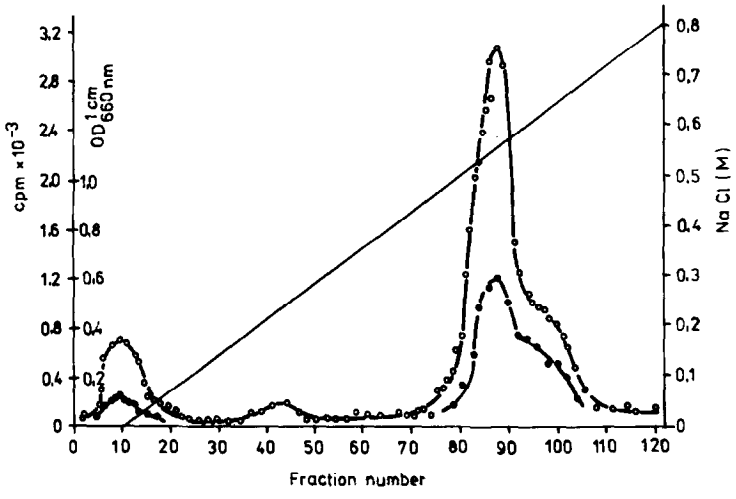


Fig. 1. Elution profile of [^{14}C] acetylated pectic acid on DEAE Sephadex A-25 column with 0 - 0.8 M NaCl with 50 mM Tris-HCl buffer pH 7.4. •—• optical density after reaction with bovine serum albumin, o—o radioactivity /cpm/.

RESULTS AND DISCUSSION

The initial determination of Vi phage III deacetylase activity, using the [^{14}C] acetylated pectic acid as a substrate, was carried out according to methods previously described / 5,6 /. Inherent in the results obtained was a high error, especially for low phage concentrations. This error was the result of a high specific radioactivity of the blind test / without phage /. It indicated a low efficiency of the polyhexuronic acid precipitation by uranyl nitrate. In the further experiments n-butanol was used for the extraction of the liberated acetic acid. The separation coefficient in water : n-butanol system was $\alpha = 1$ / Fig. 2 /, and the radioactivity of the n-butanol fraction after the extraction of the water solution of [^{14}C] acetylated pectic acid did not surpass 0.3%. On the basis of those data the determination of deacetylase activity was carried out as follows: To 0.1 ml of [^{14}C] acetylated pectic acid in a con-

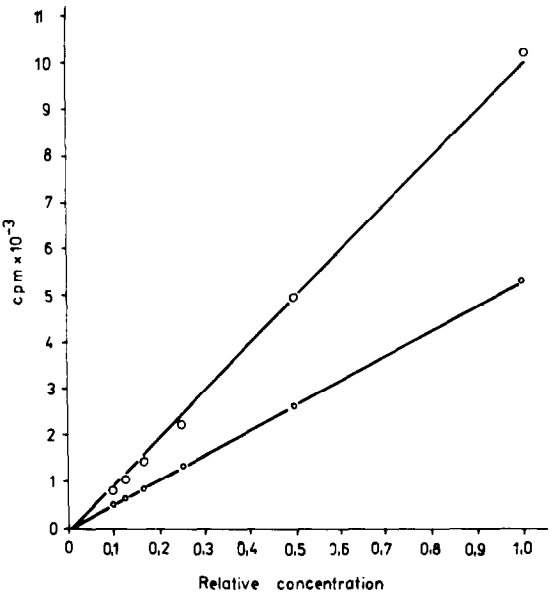


Fig. 2. Radioactivity of $CH_3{}^{14}COOH$ in water (o—o) and after extraction with n-butanol (o—o), as a function of relative concentration of the acetic acid. To 0.4 ml of $CH_3{}^{14}COOH$ with a known radioactivity 0.4 ml of n-butanol was added. After 15 min. extraction at room temperature, the radioactivity of the organic phase was measured.

centration of 6 mg/ml in 67 mM phosphate buffer pH 7.8, an equal volume of phage suspension of given concentration in the same buffer was added. After 20 min. incubation at 37°C the reaction was stopped by adding 0.05 ml of 1 M acetic acid and by cooling the mixture in an ice bath. Afterwards 0.4 ml of n-butanol was added and the extraction of the acetic acid was carried out for 15 min. at room temperature. After centrifugation / 500 x g, 1 min. / the radioactivity of the 0.3 ml n-butanol fraction was measured.

Using this method the relationship between the radioactivity of the liberated acetic acid and concentration of Vi phages I, II and III was determined / Fig. 3 /. The dependence found for the Vi phage III deacetylase, taking into account the efficiency of titration on Citrobacter Ci 23

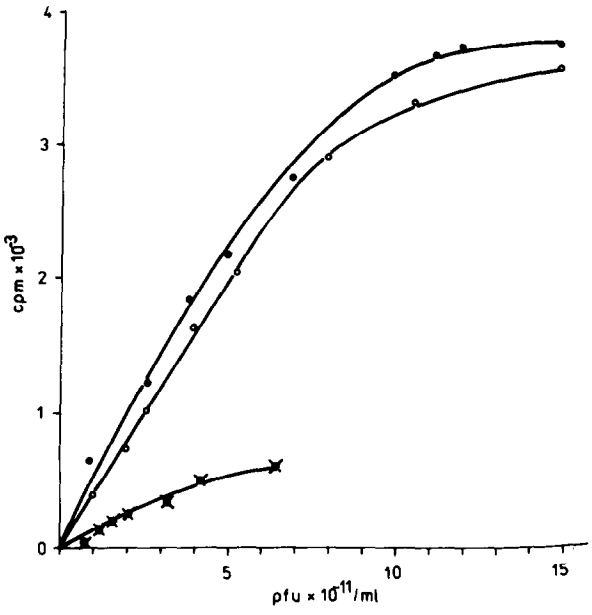


Fig. 3. Radioactivity of released acetic acid from acetylated pectic acid as a function of concentration of Vi phages. The phages were inoubated with [^{14}C] acetylated pectic acid in a concentration of 6 mg/ml in 67 mM phosphate buffer pH 7.8, for 20 min. at 37°C. •—• Vi phage I, ▪—▪ Vi phage II, o—o Vi phage III.

- S. typhi, is in accordance with that obtained earlier / 6 /. The dependence is of a similar nature for Vi phage I, too. An explicit enzymatic activity reduction for Vi phage II could have been caused by a low specificity of the enzyme to this substrate, or by some inhibitory effect of the phosphates on the reaction, as has been suggested by Taylor / 3 / who investigated the receptor activity of Vi polysaccharide. In Table 1 the results of deacetylase activity of Vi phage II in various media are shown. The highest activity of Vi phage II enzyme can be shown in Tris-HCl buffer. An addition of the phosphate buffer to this medium decreased the activity of Vi phage II deacetylase. In 80 mM phosphate buffer the activity of this phage enzyme decreases to about 20% of the initial activity / Fig. 4 /. Those results can substantiate

Table 1

Relationship between Vi phage II deacetylase activity and the buffer system / 50 mM, pH 7.8 /.

Buffer	Radioactivity / cpm /	% of radioactivity
Tris-HCl	1660	100
ammonium acetate	1426	86
phosphate	900	54

Vi phage II were in a concentration of about 3×10^{11} pfu/ml. Other conditions of reaction were the same as described previously.

the low activity of the Vi phage II deacetylase in a 67 mM phosphate buffer / cf Fig. 3 /. The optimum pH of deacetylase activity of Vi phage I, II and III is similar / Fig. 5 /, its value being 7.8. This result additionally confirms that

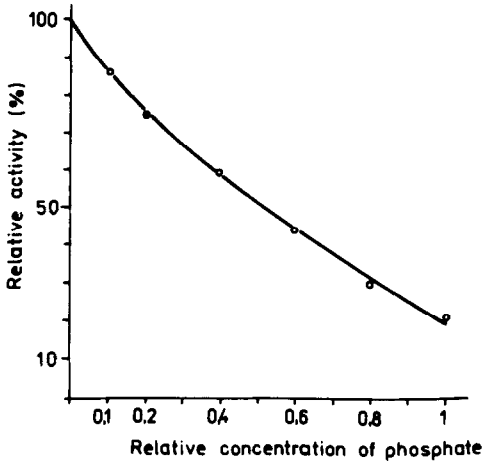
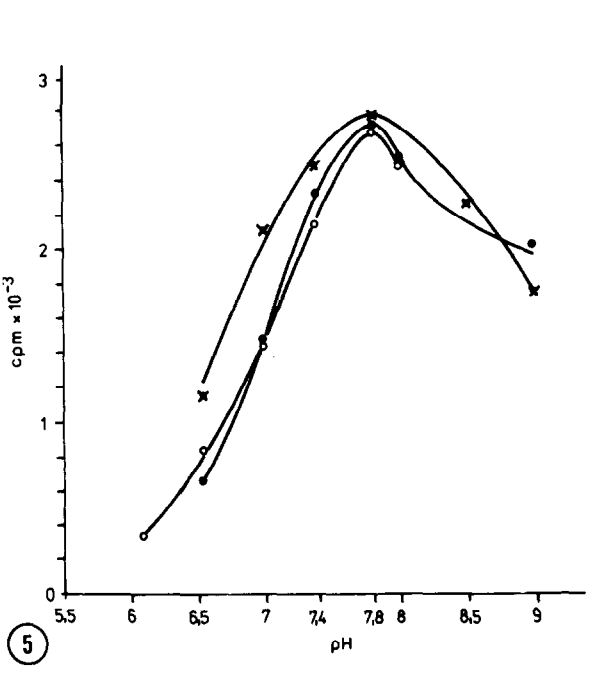


Fig. 4. Dependence of Vi phage II deacetylase activity on the concentration of phosphate in 80 mM Tris-HCl buffer pH 7.8. Relative concentration of phosphate in incubation medium was calculated from the formula:

$$\frac{\text{80 mM phosphate buffer pH 7.8 /ml/}}{\text{80 mM phosphate buffer pH 7.8 /ml/ + 80 mM Tris-HCl buffer pH 7.8}}$$

Concentration of substrate, time of incubation and temperature were the same as previously described / cf Fig. 3 /, concentration of phages - about 2×10^{11} pfu/ml.



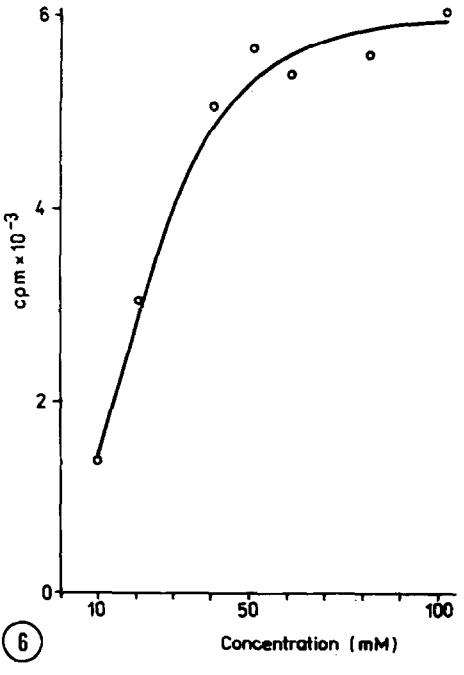


Fig. 5. Relationship between Vi phages deacetylase and the pH. All phages were in a concentration about 2 x 10^{11} pfu/ml. The reaction was proved in 50 mM Tris-HCl buffer. ●—● Vi phageI, ⊠—⊠ Vi phage II, o—o Vi phage III.

Fig. 6. Dependence of Vi phage II deacetylase activity on the molarity of Tris-HCl buffer pH 7.8. Vi phage II were in a concentration about 2 x 10^{11} pfu/ml.

the decrease in deacetylase activity of Vi phage II is caused by phosphate.

Nextly, the dependence of the deacetylase activity of Vi phage II on the molarity of Tris-HCl buffer / Fig. 6 /, and the relation between amount of liberated radioactivity and phage concentration were determined / Fig. 7 /. This results prove that the acetylated pectic acid is a substrate for deacetylase of all the Vi phages. Probably the suppression of deacetylation reaction of the Vi phage II enzyme by the phosphate buffer indicates an allosteric nature of this enzyme / study in progress /.

In the second part of this work, experiments showing the receptor activity of acetylated pectic acid were car-

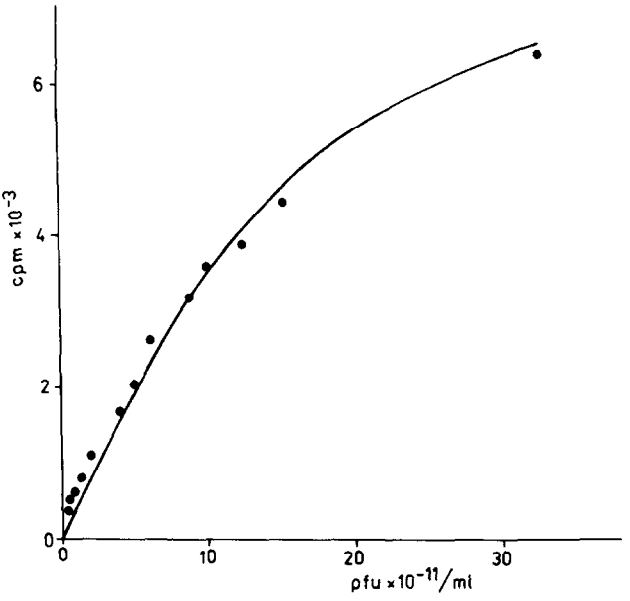


Fig. 7. Relationship between Vi phage II catalysed acetic acid liberation from acetylated pectic acid and the concentration of phage. Increasing amounts of phages were incubated in 50 mM Tris-HCl buffer pH 7.8, containing acetylated pectic acid / 6 mg/ml /, at 37°C.

ried out. Methods for the determination of receptor activity of Vi polysaccharide were used / 1,2,14 /. In these experiments blood cell membranes were coated with acetylated pectic acid or with Vi polysaccharide. After incubation and centrifugation of the erythrocyte membrane / 3000 x g, 10 min. / the concentrations of Vi polysaccharide and acetylated pectic acid in the supernatant were determined. The concentration of Vi polysaccharide in the supernatant was 90% lower, but the concentration of the acetylated pectic acid was the same as the control. We concluded that in the conditions used the erythrocyte membranes are not coated by the acetylated pectic acid. This makes it impossible to show the specificity of Vi phage adsorption in this artificial system, and to determine the receptor activity of the acetylated pectic acid.

ACKNOWLEDGEMENTS

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