An Exopectate Lyase of *Butyrivibrio fibrisolvens* from the Bovine Rumen

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(Received 15 December 1981; revised 22 April 1982)

An extracellular pectinolytic enzyme produced by *Butyrivibrio fibrisolvens* isolated from the bovine rumen was studied. The enzyme had a pH optimum of 8.0 to 8.5 and was stimulated by Ca^{2+} and inhibited by EDTA. The products of pectinolysis had an absorption peak at 235 nm and reacted with thiobarbituric acid, indicating a lyase type of action. The enzyme cleaved the substrates terminally from the reducing end; action on poly- and oligogalacturonates resulted in the formation of an unsaturated trigalacturonate. The enzyme was classified as an exopectate lyase (EC 4.2.2.9). A pectinesterase was also produced by *B. fibrisolvens* but polygalacturonase was not detected.

INTRODUCTION

Butyrivibrio fibrisolvens is one of the more important bacterial species in the rumen and is well adapted to the breakdown of plant cell wall polysaccharides. Its pectinolytic properties were studied by Dehority (1969) and Gradel & Dehority (1972) who showed that *B. fibrisolvens* degraded pectin from grass. Clarke *et al.* (1969) found differences between the decomposition of pectin by *B. fibrisolvens* and by *Lachnospira multiparus*.

Our preliminary investigations indicated that the pectinolytic enzyme of *B. fibrisolvens* differed in some respects from the enzymes of other rumen bacteria. This paper presents more detailed information on the activity of the enzyme.

METHODS

Organisms and growth. Three strains of *Butyrivibrio fibrisolvens* were isolated from the rumen of a cow with a permanent rumen fistula, using a selective medium with pectin as the only source of energy. Identification was by microscopy and physiological and biochemical tests. Growth in liquid pectin medium was abundant with a heavy flocculent sediment and the cells formed long filaments. Fermentation products of pectin were formic, acetic, butyric and lactic acids. The most actively pectinolytic strain (strain 781) was selected for study.

Growth medium and culture conditions were those described by Wojciechowicz & Ziolecki (1979). The organism was grown in 10 litre batches.

Separation of the enzyme. The culture fluid was centrifuged and enzymes from the supernatant were salted out with $(\text{NH}_4)_2\text{SO}_4$ as described by Wojciechowicz (1971). The precipitate was dissolved, dialysed, freeze-dried and the resulting preparation was designated S_{2L}.

A part of S_{2L} was redissolved, further desalted by gel filtration through Sephadex G-25, freeze-dried and designated S_{2LS}. The other part was subjected to chromatography on a DEAE-cellulose column (1 × 45 cm) equilibrated with 0.01 M-Tris/HCl buffer, pH 7.6. The enzyme was eluted by stepwise addition of 0.02, 0.2 and 1.0 M-Tris/HCl buffer, pH 7.6, and 5 ml fractions were collected. The activity of the fractions was examined by absorbance at 235 nm of an incubation mixture prepared as described below for enzyme assays. The active fractions eluted at 0.02 M-Tris/HCl; they were combined, dialysed, concentrated with Aquacide (mol. wt 250000) and designated S_{2LC}.

Substrates. Polygalacturonic acid (no. 96723) was from Th. Schuchardt, Munich, Germany; citrus pectin N.F. (C grade, lot 33335) was from Calbiochem and sodium polypectate (no. 5918) was from Serva, Heidelberg, Germany. Oligogalacturonic acid of polymerization $n = 10$ was prepared according to McCready & Seegmiller

(1954). Saturated oligogalacturonic acids of polymerization $n = 2$ to $n = 7$ were prepared according to Rexova-Benkova (1970) and unsaturated di- and trigalacturonic acids were obtained as described earlier (Wojciechowicz, 1972).

Enzyme assays. Analytical methods were as previously described (Wojciechowicz & Ziiolecki, 1979; Wojciechowicz *et al.*, 1980). The typical reaction mixture contained 0.5% (w/v) polygalacturonate or pectin in 0.05 M-Tris/HCl buffer, pH 8.0, 1 mM-CaCl₂ and enzyme preparation. In some experiments acetate buffer, pH 5.6, or phosphate buffer, pH 6.6, were used. The reaction was carried out at 30 °C and absorbance of the reaction mixture was measured at 235 nm, or in the range 215 to 265 nm, after dilution of 0.2 ml of the reaction mixture with 2.5 or 5.0 ml of 0.01 M-HCl.

Liberation of aldehyde groups was estimated spectrophotometrically using the Nelson-Somogyi reagent (Somogyi, 1952).

Chromatography of degradation products of polygalacturonate and pectin was on Whatman no. 1 paper as described by Wojciechowicz & Ziiolecki (1979).

Separation of degradation products of oligogalacturonates was by TLC on silica gel sheets Silufol (Kavalier, Votice, Czechoslovakia) in butan-1-ol/formic acid/water (2:3:1, by vol.) according to Koller & Neukom (1964). Oligogalacturonates were detected by the aniline phthalate reagent (Partridge, 1949). Unsaturated products were revealed by the method of Warren (1960) with thiobarbituric acid reagent. Distribution of unsaturated bonds in the reaction products was estimated according to MacMillan *et al.* (1964) and Sato & Kaji (1976). The activity of pectinesterase was estimated by the cup-plate technique of Reid (1950).

RESULTS

Effect of pH, Ca²⁺ and EDTA on enzyme activity

The optimum pH for enzyme activity towards polygalacturonic acid was determined by measuring total absorbance at 235 nm of reaction mixtures buffered at different pH values. The buffer systems used were those described by Wojciechowicz & Ziiolecki (1979) and covered the pH range of 5.5 to 10.0. The activity was maximum at pH 8.0 to 8.5.

The effect of CaCl₂ (0 to 1.25 mM) on enzyme activity was examined with polypectate as substrate. CaCl₂ stimulated enzyme activity up to the concentration of 0.75 mM but with higher concentrations the effect was less (Fig. 1). Incubation in the presence of 3 mM-EDTA resulted in an almost complete inhibition of activity (Fig. 1).

Substrate specificity

The activity of the enzyme, as measured by the increase of absorbance during 90 min incubation, was about 10% greater with polygalacturonate as the substrate than with pectin. There was a lowering in the initial rate of degradation of polygalacturonic acid and oligogalacturonates as the chain length decreased (Table 1). Tetragalacturonic acid appeared to be the smallest oligogalacturonate that could be degraded; there was no detectable breakdown of trigalacturonate.

Viscosity changes

As compared to the pectinolytic activity of other rumen bacteria, e.g. *Bacteroides rumenicola*, changes in the viscosity of a 0.5% (w/v) pectin solution produced by the enzyme of *B. fibrisolvens* under standard conditions (Wojciechowicz, 1971) were very slow. After 10 min incubation the decrease in viscosity with *B. rumenicola* enzyme was 79% while with *B. fibrisolvens* it was only 16% and it took the latter 2 h to produce a 63% drop in viscosity. Different amounts of enzyme preparation of either organism were used in the comparative test to compensate for the difference in specific activity, which was greater in *B. rumenicola*, and to obtain a similar rate of breakdown as measured by the increase of UV absorbance. Thus, the difference in the rate of reduction of viscosity was due not to the extent but rather to the mode of degradation, random in *B. rumenicola* and terminal in *B. fibrisolvens*.

Distribution of unsaturated bonds in reaction products

Attack at the reducing end of the substrate molecule should result in unsaturated degradation products and the absence of a double bond on the chain after the unsaturated units have been removed from the molecule. The absorbance values of the shorter chain products were in good agreement with those of the unfractionated reaction mixture (Fig. 2). The results indicated that

Fig. 1

Fig. 1. Effect of Ca^{2+} and EDTA on enzyme activity. Reaction mixtures contained 0.5% (w/v) polygalacturonate in 0.05 M-Tris/HCl buffer, pH 8.0 and 0.62 mg of enzyme preparation (S_{21}) per ml with (○) or without (●) 3 mM-EDTA. Incubation time was 60 min at 30 °C.

Fig. 2

Fig. 2. Absorbance at 235 nm of original reaction mixture (●) and of its fractions representing the shorter (○) and longer (△) chained products. Reaction mixtures contained 0.5% (w/v) polygalacturonate in 0.05 M-Tris/HCl buffer, pH 8.0, 1 mM- CaCl_2 and 2.5 mg of enzyme preparation (S_{21}) per ml. Incubation temperature was 30 °C.

Table 1. *Rate of degradation of pectate and oligogalacturonates by the enzyme of B. fibrisolvens*

Substrate (0.5% sodium pectate in 0.05 M-Tris/HCl or 1 μmol of oligogalacturonates in 1 ml 0.05 M-Tris/HCl at pH 8.0) was incubated with 0.05 ml of enzyme solution (1 mg enzyme preparation S_{21S}) and incubated for 10, 20, 30, 40 and 60 min at 30 °C. Reducing values were estimated with the Nelson-Somogyi reagent.

Substrate	Rate of degradation (mmol g ⁻¹ s ⁻¹)
Sodium pectate	1.4×10^{-2}
Polygalacturonate*	5.0×10^{-3}
Heptagalacturonate	4.2×10^{-3}
Hexagalacturonate	4.0×10^{-3}
Pentagalacturonate	8.2×10^{-4}
Tetragalacturonate	2.1×10^{-4}
Trigalacturonate	0

* Degree of polymerization $n = 10$.

the enzyme removed terminal unsaturated trimer units from the reducing end of polygalacturonate chains.

Degradation products

The products formed from polygalacturonate were detected by paper chromatography (Fig. 3). A spot of unsaturated trimer appeared in the early phase of incubation. With prolonged incubation the chromatogram revealed very weak spots of some other oligogalacturonates, probably saturated dimer and saturated trimer. The saturated monomer which should have

Fig. 3. Densitometric illustration of spots on paper chromatograms of degradation products formed from polygalacturonate by enzyme action for various times. Reaction mixtures contained 0.5% (w/v) polygalacturonate in 0.05 M-Tris/HCl buffer, pH 8.0, 1 mM-CaCl₂ and 1.7 mg of enzyme preparation (S_{21,C}) per ml. The spots were visualized by alkaline AgNO₃. Standards: GA, saturated monomer (galacturonic acid); u-2GA and u-3GA, unsaturated dimer and trimer, respectively.

resulted from decomposition of the tetramer was probably produced in amounts too small to be detected.

The trimer was not degraded further. The tetramer was cleaved at the third bond from the reducing end of the chain to yield an unsaturated trimer and a monomer. The pentamer was split into an unsaturated trimer and a dimer and hexamer into an unsaturated trimer and a trimer. The heptamer was cleaved first into an unsaturated trimer and a tetramer, the latter then being degraded into an unsaturated trimer and a monomer. Degradation of the oligomers leading invariably to an unsaturated trimer and a saturated moiety also suggests that action of the enzyme was from the reducing end.

No degradation products were found after incubation of polygalacturonate with the enzyme preparation S_{2LS} in acetate and phosphate buffers at pH 5.5 and 6.6 at 30 °C for 24 h. This indicates that no D-galacturonase (EC 3.2.1.15) was present in the enzyme preparation.

The cup-plate test of Reid (1950) for the presence of pectinesterase (EC 3.1.1.11) in the enzyme preparation S_{2LS} was positive.

DISCUSSION

The results presented in this paper seem to indicate that *B. fibrisolvens* produced a terminally acting polygalacturonate lyase, which we have attributed to an exopectate lyase (EC 4.2.2.9). Enzymes of this type releasing an unsaturated trimer seem to occur rarely in Nature and so far have been found only in *Streptomyces nitrosporeus* (Sato & Kaji, 1977). An unsaturated trimer as the major end-product of pectin degradation resulted also from the action of endopectate lyase (EC 4.2.2.2) of a *Bacillus* sp. from soil (Hasegawa & Nagel, 1966) and of *Bacillus pumilus* (Dave & Vaughn, 1971).

Butyrivibrio fibrisolvens also seems to be unique in this respect among the rumen bacteria as all

the other major pectinolytic species so far examined [*Bacteroides ruminicola* (Wojciechowicz, 1971), large and small treponemes (Wojciechowicz & Ziotecki, 1979; Ziotecki & Wojciechowicz, 1980) and *Lachnospira multiparus* (Wojciechowicz *et al.*, 1980)] produced mainly endopectate lyases randomly cleaving the polygalacturonate molecule to yield mainly unsaturated digalacturonate and unsaturated galacturonate together with some saturated oligogalacturonates as end-products.

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