The Pentosanases of some Rumen Bacteria

By B. H. HOWARD, G. JONES AND MARY R. PURDOM

The Rowett Research Institute, Bucksburn, Aberdeen

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A study of the hydrolysis of some soluble pentosans by suspensions of sheep-rumen bacteria was previously reported (Howard, 1957). It was shown that the mixture of bacteria normally present in the rumen is able, by removal of arabinose side chains if present, and by apparently random breaking of the xylan chain, to hydrolyse the pentosans completely into their constituent monosaccharides. The next step in this investigation was to examine pure cultures of those organisms of the rumen flora which are active in pentosan decomposition. From a number of such cultures which have been isolated in this Laboratory, two were selected for detailed study. One was *Bacteroides amylogenes* (Doetsch, Howard, Mann & Oxford, 1957), isolated from sheep rumen on a medium containing xylose. The other, which bears the number 11 in our Laboratory collection, was a strain isolated from bovine-rumen contents on a medium containing wheat-flour pentosan, and tentatively assigned to the genus *Butyrivibrio* (Bryant & Small, 1956). A brief description of this organism is given in the Appendix to this paper.

Preliminary experiments showed that intact cells from pentosan cultures of each of these bacteria vigorously hydrolysed the pentosan, and attention was then turned to the preparation of active cell-free materials. The properties of such preparations are the subject of this paper. Attempts have been made to separate the component enzymes of the pentosan-hydrolysing complex, and to determine their properties and some of the factors controlling the production of them by the bacteria.

MATERIALS AND METHODS

Bacterial cultures. The strain of *Bacteroides amylogenes* used was that described by Doetsch *et al.* (1957). The *Butyrivibrio* sp. was isolated in 1957 from the rumen of a heifer fed on silage. Both cultures used in this work were obtained from freeze-dried samples prepared soon after the original isolations. Stock cultures were maintained on the medium described below, solidified with 2% of agar and containing xylose instead of the wheat-flour pentosan. Subcultures were made once a month and stored in the refrigerator. Cultures were incubated at 38°.

The bacteria from two 2-day slope cultures on pentosan medium, suspended in sterile 0.9% NaCl, were used as inoculum for each 250 ml. of liquid culture.

Preparation of media. The following stock solutions were

used in preparing the culture media: I, 0.3% K_2HPO_4 . II, A solution containing 0.3% of KH_2PO_4 , 0.6% of $(NH_4)_2SO_4$, 0.6% of NaCl, 0.06% of $MgSO_4 \cdot 7H_2O$ and 0.06% of $CaCl_2$. III, A solution of 0.625% of cysteine hydrochloride and 6% of $NaHCO_3$. IV, 2.5% (w/v) wheat-flour pentosan. Solution III was sterilized by passage through a Seitz filter and solution IV by steaming for 30 min. on 3 successive days.

Bottles of 300 ml. capacity, containing 250 ml. of liquid medium, were used for large-scale culture. Into each bottle were placed solutions I and II (37.5 ml. each), Difco yeast extract (1.25 g.), strained rumen liquor (25 ml.), 0.1% solution of resazurin sodium (L. Light and Co. Ltd.; 0.25 ml.) and water (79 ml.). The mixture was sterilized by heating the bottles in the autoclave for 15 min. at 15 lb./in.² pressure and, after cooling, was saturated with sterile CO_2 . Finally, sterile solution III (21 ml.) and sterile solution IV (50 ml.) were added with sterile precautions. The technique of Hungate (1950) was used throughout this work, i.e. the cultures were always grown in rubber-stoppered tubes or bottles under an atmosphere of sterile, O_2 -free CO_2 , and a flow of this gas into the tube or bottle was maintained whenever one was opened.

Carbohydrate materials. The following substances were used. (1) The soluble pentosan (araboxylan) from wheat flour. The ratio xylose:arabinose in the wheat-flour pentosan was about 1.77:1. (2) Rhodymenan, the soluble xylan of the red seaweed *Rhodymenia palmata*. The preparation of these was described by Howard (1957). (3) Xylan (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). This material as purchased contained an appreciable amount of arabinose and a little glucose and galactose. It was used without further purification in culture media, but for use as a substrate in enzyme experiments it was purified once through the copper complex as described by Sørensen (1957). The ratio xylose:arabinose in the product was 12:1. (4) The series of β -1:4-linked xylose oligosaccharides, from xylobiose to xylohexaose, separated from a partial hydrolysate of wheat-flour pentosan (Howard, 1957). Xylobiose was crystalline; the other four materials were chromatographically homogeneous amorphous solids or syrups. (5) The mixture of arabinose- and xylose-containing oligosaccharides obtained from the same hydrolysate as mentioned in (4) above, and referred to as fraction 12 in Table 1 of Howard (1957). This material, which was eluted during chromatography on charcoal by 30% (v/v) ethanol, had a mean degree of polymerization (DP) of 11.6, and contained no oligosaccharides of DP less than 6. Complete acid hydrolysis yielded approximately equal amounts of xylose and arabinose. Hydrolysis with *n*-acetic acid at 100° for 4 hr. (Professor I. A. Preece, personal communication) yielded much arabinose and traces of xylobiose and xylotriose as the only chromatographically identifiable substances. This material will be

referred to in this paper as 'mixed oligosaccharides' to distinguish it from the other oligosaccharides composed only of anhydroxylose units. (6) D-Glucose, L-arabinose and D-xylose were AnalaR or Kerfoot's Biochemical Reagents. For use in culture media all carbohydrates were sterilized separately in 2.5% (w/v) solution or suspension in water, polysaccharides by intermittent steaming (see above), simple sugars by filtration through Berkefeld candles and xylobiose by filtration through a small sintered-glass filter, grade 5/3.

Preparation of freeze-dried powders from bacterial cultures.

(a) Large-scale preparation from cells only. After an appropriate period of incubation, usually about 5 days, the bottles containing the cultures were transferred to the refrigerator and left overnight. Residual pentose and pH were measured, and a Gram-stained smear was examined microscopically to check the purity of the culture. The cells were sedimented by centrifuging at 0° at 22 000 g for 30 min. The sediment, after draining as free as possible of culture fluid, was suspended in water with the aid of a glass homogenizer. Sufficient butanol to make two layers was added with shaking, the mixture stored at 0° overnight and then centrifuged at 0° at 22 000 g for 30 min. The clear aqueous layer was removed, the butanol and bacterial debris were dispersed in a little water and a second aqueous extract was obtained by centrifuging. The combined extracts, having a volume of about one-tenth that of the original culture, were filtered through a sintered-glass filter, grade 5/3, and dialysed, first against running tap water for 36 hr. and finally overnight at 1° against distilled water. The contents of the dialysis sacs were distributed as layers less than 1 cm. deep in glass beakers, and freeze-dried. Disks of filter paper, fastened over the beakers with rubber bands, prevented loss of the powder on opening the machine. The product was a very fluffy, pale buff-coloured powder, readily soluble in water to a pale-brown solution. One litre of culture yielded about 50 mg. of powder.

(b) Small-scale preparation from whole cultures. When the bacteria were grown on diffusible carbohydrates it was possible to obtain preparations containing both the enzymes excreted into the medium and those associated with the cells. To the culture, grown in a small screw-capped bottle, was added enough butanol to make two layers. The bottle was well shaken and stored in the refrigerator overnight. The subsequent procedure was the same as described above, and yielded between 20 and 30 mg. of freeze-dried powder from 15 ml. of culture.

Preparation and storage of enzyme solutions. The freeze-dried powder was incubated with the appropriate buffer solution for 30 min. and the trace of insoluble matter removed by centrifuging. The clear supernatant was removed and, if not required for use immediately, was stored in the refrigerator under toluene. Except in the electrophoretic experiments, McIlvaine's (1921) phosphate-citrate buffers were used throughout this work.

Measurement of enzyme activity. (i) Xylanase. Wheat-flour pentosan or xylan was used in this assay. The former substrate (200 mg.) was dissolved in water (100 ml.) to yield a slightly opalescent solution which was stable for a few weeks in the refrigerator. A solution of the xylan was prepared by the method of Sørensen (1957). Unlike the xylan used by Sørensen, that used in this work formed a copious precipitate when the alkaline solution was dialysed. A turbid solution containing about half the

starting material was obtained after centrifuging. The proportions of arabinose, glucose and galactose in an acid-hydrolysed portion of this solution were no greater than in the purified xylan. After determination of the total pentose concentration in the xylan solution, it was diluted with water to a concentration of 2 mg./ml. (as xylose) and stored in the refrigerator. Both polysaccharide solutions were filtered through Whatman no. 1 paper before use. Enzyme assay was carried out in tubes marked at 25 ml. Each tube contained polysaccharide solution (0.5 ml., i.e. about 1 mg. of substrate), together with enzyme and buffer solution to a total vol. of 1 ml. The tubes, and appropriate controls, were placed in a water bath at 38° for 4 hr. A portion (1 ml.) of the alkaline copper reagent of Nelson (1944) was added and reducing sugar estimated colorimetrically. The reducing sugar was expressed as xylose after correction for the small reducing power of the polysaccharides when incubated in the absence of enzyme, and the result used to calculate the apparent percentage hydrolysis of the xylan or pentosan. It was found unnecessary to deproteinize the solutions before carrying out the reducing-sugar determination.

(ii) Xylobiase. The procedure was similar to that in (i) above. Each tube contained xylobiose (0.5 ml. of aqueous 0.01% solution, i.e. 50 µg.), and incubation was for 1 hr. only. The reducing value of the solutions was corrected for that of xylobiose alone, determined in a control tube without enzyme.

(iii) 'Cellulase' and cellobiase. Our colleague, Dr G. N. Festenstein, kindly tested some of our materials for hydrolytic activity against sodium carboxymethylcellulose and cellobiose. Hydrolysis of the former substrate was measured by a reducing-sugar method, hydrolysis of the latter by a specific test for glucose employing glucose oxidase (Festenstein, 1958a, b).

Qualitative test for enzyme activity. Approximately equal volumes of substrate solution (2%, w/v) and buffered enzyme solution were incubated under toluene in sealed capillary tubes (Porter & Hoban, 1954) for between 18 and 48 hr. The solution was then transferred to paper which had been treated with HgCl₂ (Bealing & Bacon, 1953), and chromatograms were prepared with the techniques and solvent systems described previously (Howard, 1957). It was occasionally necessary to examine the products of hydrolysis of polysaccharides when the hydrolysis had proceeded to only a small extent. In these cases the solution to be tested was treated with 2 vol. of ethanol, and the precipitated polysaccharide removed by centrifuging. The supernatant solution was then deionized and concentrated for paper chromatography.

Thermal-stability experiments. A tube containing buffer solution (10 ml., pH 6.5) was deeply immersed in a water bath maintained at 60°. After allowing a period for equilibration of temperature, a solution of the enzyme in the same buffer solution (2 ml.) was quickly added and mixed, and a sample (2 ml.) was immediately transferred to a tube chilled in ice. Further samples were withdrawn while the enzyme solution was kept at 60°. The chilled samples of heated enzyme were stored under toluene in the refrigerator until tested for activity.

Electrophoretic methods. Zone electrophoresis was in columns of starch; the apparatus used has been described by Mould & Thomas (1958) and Mould & Stephen (1958). 0.05 M-Sodium phosphate buffer, pH 6.5 or 7.5, was used,

Table 1. *Enzyme activity of preparations from two strains of rumen bacteria after growth on various carbohydrates*(a) *Bacteroides amylogenes*; (b) *Butyrivibrio* sp. +, Enzyme activity present; 0, enzyme activity absent; —, not tested.

Growth substrate	Substrates for enzyme tests												
	Xylobiose		Xylan		Wheat-flour pentosan*				'Mixed oligosaccharides'*				
					(a)		(b)		(a)		(b)		
	(a)	(b)	(a)	(b)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	
Glucose, arabinose, xylose	0	0	0	0	0	0	0	0	0	0	0	—	—
Xylobiose	+	—	0	—	0	+	—	—	+	+	—	—	—
Wheat-flour pentosan	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhodymenan	+	—	+	—	+	+	—	—	+	+	—	—	—
Xylan	—	+	—	+	—	—	+	+	—	—	+	+	+

* The two symbols for each entry in these columns refer to (1) the breaking of xylose-xylose linkages, and (2) the breaking of arabinose-xylose linkages.

and a p.d. of 500 v was applied to the electrodes. In some experiments the column was cooled by a flow of water at 1° through the jacket, in others no temperature control was applied and the column was at, or a little above, room temperature (about 17°).

Other methods. Pentoses were determined by a modification of Drury's (1948) method, and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Chromatography on charcoal was as described by Howard (1957).

RESULTS

Effect of growth medium on production of enzymes. The two strains of bacteria were grown on media containing different substances as sole source of fermentable carbohydrate. When the carbohydrate was a polysaccharide the final preparation contained the enzymes associated only with the bacterial cells [method (a) above]; when growth was on a diffusible carbohydrate, the final preparation contained also any enzymes secreted into solution [method (b) above]. The effects of these products on xylobiose, wheat-flour pentosan, xylan and 'mixed oligosaccharides' were determined. Hydrolytic action was assessed quantitatively by measuring the increase in reducing power, and qualitatively by paper chromatography. The results are summarized in Tables 1 and 2. The preparations which decomposed xylobiose had a solely hydrolytic action when the concentration of the substrate was 1 or 2%. Enzyme preparations from cultures grown on the polysaccharides hydrolysed the wheat-flour pentosan and the xylan in all cases tested. Xylose and small amounts of xylose-oligosaccharides were present in the hydrolysates; the wheat-flour pentosan and 'mixed oligosaccharides' hydrolysates also contained some arabinose. In experiments with the latter substrate rupture of xylose-xylose linkages was detected by the formation of xylose or chromatographically detectable oligosaccharides or both.

Table 2. *Activity of enzyme preparations from two strains of rumen bacteria*

Activity is expressed as µg. of xylose released from 1 mg. of substrate in 1 hr. by 1 mg. of enzyme powder, when incubated under the conditions described in the text.

(a) *Bacteroides amylogenes*; (b) *Butyrivibrio* sp.

Growth substrate	Substrates for tests			
	Xylan		Wheat-flour pentosan	
	(a)	(b)	(a)	(b)
Glucose, arabinose, xylose	<1	<1	<1	<1
Xylobiose	<1	—	10	—
Wheat-flour pentosan	30	—	220	160

The clear supernatant solutions obtained by centrifuging the cultures when grown on the polysaccharides, and which contained the unused substrate, were usually discarded. A little enzyme activity was present in these solutions, such that traces of xylose, arabinose and oligosaccharides could be produced on incubation with wheat-flour pentosan. The low activity, and the presence of large amounts of unused polysaccharide substrate, rendered unprofitable any further study of the enzymes secreted into solution by the bacteria.

Electrophoretic experiments. Enzyme preparations from cultures of both bacterial strains grown on wheat-flour pentosan, rhodymenan and xylan were examined. No significant differences were found in the behaviour of the enzymes in the preparations. Whether the pH of the solution was 6.5 or 7.5 and whether the temperature was 1° or 17° did not affect the movement of the enzymes. Whatever the source of crude enzyme, and whatever the conditions, all the enzyme activity moved towards the anode. When the ability of the eluate fractions to hydrolyse xylobiose or wheat-flour pentosan was examined it was seen that at least two different enzymes were present, and that they

were partially separated by electrophoresis (Fig. 1). Some enzymically inert protein moved ahead of the more mobile enzyme, and the greatest enzyme activity per unit weight of protein was found in the least mobile fractions. Portions of the eluates were incubated with wheat-flour pentosan and xylobiose, and the resulting sugars examined chromatographically. The more mobile fractions attacked both substrates, and produced from the pentosan substantial amounts of xylose and arabinose, as well as smaller quantities of oligosaccharides. The less-mobile enzyme fractions yielded less monosaccharide but the proportion of arabinose to xylose became higher. The least-mobile fractions produced no monosaccharides but yielded traces of xylobiose and other oligosaccharides. Appropriate fractions from the electrophoretic separation were pooled to give two solutions, one containing most of the xylobiase activity with a trace of the xylanase, the other containing most of the xylanase, free from xylobiase. A portion of the xylanase solution was freed from buffer salts by dialysis and freeze-dried. A white powder resulted which was much less readily soluble in water than the original crude enzyme. When subjected to electrophoresis under the same conditions as before only a single peak of xylanase activity was observed. Some tailing of enzyme occurred.

Properties of xylobiase

Sources. Three sources of this enzyme were available: the products of electrophoresis of crude enzyme from cultures of *Bacteroides amylogenes* and the *Butyrivibrio* sp. on wheat-flour pentosan, and the crude enzyme from the culture of *Bacteroides amylogenes* on xylobiose. These will be referred to as A, B and C respectively. A and B were slightly contaminated with xylanase, C was not. In some experiments the presence of the xylanase was immaterial, but in others it was necessary to use

completely xylanase-free material. In such experiments only xylobiase C was used. No difference in properties between the xylobiases of the two bacterial strains was observed.

Action pattern. Chromatographic tests showed this enzyme to be active not only against xylobiose but against all the other xylose-oligosaccharides, at least up to xylohexaose. When the concentration of xylobiose was 1 or 2% (w/v) the action of the enzyme appeared to be purely hydrolytic, no sign of transferring activity being apparent at any stage of the reaction. Xylobiose was progressively, and at length completely, converted into xylose. Xylotriose yielded xylobiose and xylose, initially in roughly equal proportions, but later, when all but a trace of substrate had been decomposed, most of the xylobiose had also been hydrolysed. The first products of action on xylotetraose were xylotriose and xylose; xylobiose did not appear until later in the reaction and was always a minor constituent of the mixture. When nearly all the xylotetraose had disappeared, the mixture contained traces of xylotriose and xylobiose, and much xylose. The first products of action on xylopentaose were xylotetraose and xylose; xylotriose and xylobiose appeared successively after this, and in the early stages of hydrolysis the concentration of the intermediates always decreased in the order xylotetraose, xylotriose and xylobiose. At the end of the reaction the substances disappeared from the mixture in the order xylopentaose, xylotetraose, xylotriose and xylobiose. A similar train of events was observed during hydrolysis of xylohexaose.

A series of fractions from the electrophoresis of crude enzyme from *Bacteroides amylogenes* was tested for β -xylosidase activity (Conchie, 1954). (We are indebted to our colleague Dr J. Conchie for this determination.) The ability of the different fractions to hydrolyse phenyl β -xyloside closely paralleled their ability to hydrolyse xylobiose.

A portion of xylobiase C was incubated with solutions of the oligosaccharides (xylobiose to xylohexaose) in each of which the concentration was 100 μ g./ml. as xylose, pH 6.6. Reducing sugar was determined in 1 ml. samples taken at the beginning of the experiment and after incubation for 3 hr. The activity of the enzyme, as measured by the increase in reducing power of each test substance, was found to decrease slightly as the oligosaccharide series was ascended (Fig. 2).

Neither the crude enzyme preparation nor the electrophoretically prepared xylobiase (xylobiase B) from a culture of the *Butyrivibrio* sp. on wheat-flour pentosan was able to hydrolyse cellobiose. There was at least a 1000-fold difference between the cellobiase and xylobiase activities.

The xylobiase preparations A and B produced on long incubation with wheat-flour pentosan a

Fig. 1. Zone electrophoresis of *Butyrivibrio* sp. enzymes in 0.05M-sodium phosphate buffer, pH 7.5, for 19 hr. Temp., 1°; applied p.d., 500 v. O, Xylobiase activity; ●, xylanase activity. For assay conditions see text.

Fig. 2. Rates of hydrolysis of xylose oligosaccharides by enzymes of *Bacteroides amylogenes*. ○, Xylobiase; ●, xylanase. For conditions see text.

Fig. 3. Variation of activity of enzymes of *Butyrivibrio* sp. with pH. ○, Xylobiase; ●, xylanase. For conditions see text.

little xylose and a trace of arabinose. Xylobiase C, on the other hand, had a much slighter effect on the pentosan, arabinose being the only recognizable product, and had no effect at all on xylan.

pH optimum and thermal stability. Xylobiase B had an optimum pH of 6.8, when tested in phosphate-citrate buffer during 1.5 hr. against xylobiose (Fig. 3). The activity of xylobiase C was destroyed within 5 min. when heated at 60° in buffer, pH 6.5. This was demonstrated by reducing-sugar determinations after incubating the heated solutions with xylobiose for 4 hr. and confirmed chromatographically after incubating for 40 hr.

Arabinosidase activity. In addition to removing arabinose from the pentosan, the various xylobiase preparations also removed this sugar from the 'mixed oligosaccharides'. In each case xylose was the only other chromatographically identifiable substance formed. The electrophoretically prepared xylobiase fraction yielded more xylose, and xylobiase C yielded more arabinose, under identical conditions. The portion of the last-named material which had been heated at 60° for 5 min. produced arabinose but no xylose from the 'mixed oligosaccharides' and portions which had been heated for 10 min. or longer produced neither xylose nor arabinose.

Transferring activity. Xylobiose (22.6 mg.) and freeze-dried enzyme preparation from the *Butyrivibrio* sp. (2.3 mg.) were dissolved in buffer solution, pH 6.5, diluted 1:10 with water (0.1 ml.). Small drops were transferred to a HgCl₂-treated chromatogram paper at intervals during incubation for 24 hr. Development of the chromatogram showed that all but a trace of the xylobiose had disappeared during the experimental period, and that the concentration of xylose in the mixture increased throughout. In addition, small quantities of two other substances appeared transiently on the chromatogram. The major substance was evident after incubation for 1 hr., the minor substance after 2 hr., but both had disappeared after

Table 3. Charcoal-column separation of the products of action of xylobiase on xylobiose

Xylobiose (0.25 g.) was dissolved in 1.2 ml. of diluted buffer solution, pH 6.5.

Fraction no.	Ethanol in eluate (% v/v)	Wt. of fraction (mg.)	Provisional identification
1	0	—	Xylose
2	5	82	Xylobiose
3	7.5	3	Rhodymenabiase*
4	10	15	Xylotriase
5	12.5	13	3 ² -β-Xylosylxylobiase*
6	15	—	Tetrasaccharide (?)*

* Howard (1957).

24 hr. The two substances were chromatographically identical with xylotriose and 3²- β -xylosylxylobiose (Howard, 1957) respectively.

In a large-scale experiment, xylobiose (0.25 g.) and xylobiase C (8.5 mg.) were dissolved in the diluted buffer solution (1.2 ml.) and incubated for 17 hr. The resulting mixture of sugars was separated by elution with aqueous ethanol from a charcoal column and the fractions shown in Table 3 were obtained. The provisional identification of the substances is based on their chromatographic mobilities when run on the same paper as authentic material, and also on the order of their elution from the column.

Properties of xylanase

Action pattern. Enzymes attacking the xylan chain, in either xylan or wheat-flour pentosan, were less mobile than the xylobiase in the electrophoretic conditions used. No difference in behaviour between the xylanases of the two bacterial strains was observed. The more mobile of the xylanase fractions, which were contaminated with a little xylobiase (Fig. 1), yielded some xylose when acting upon xylan, and some xylose and arabinose from the pentosan. The less-mobile fractions, however, produced no monosaccharides from the pentosan and only traces of xylose from the xylan. When the enzyme in these fractions acted upon the xylan there was an initial rapid increase in reducing power, followed by a longer period during which hydrolysis was much slower. Chromatographically identifiable substances were produced only during the second stage of hydrolysis. Xylobiose was the main end product, and smaller amounts of other oligosaccharides were also formed. No chromatographically identifiable substances were formed from the pentosan; in a quantitative test, the reducing power of the enzyme-pentosan mixture became constant at a value suggesting that the pentosan had been broken down into oligosaccharides with DP of about 12.

The xylanase, like the xylobiase, acted upon the xylose oligosaccharides but in a completely different manner. A quantitative test, carried out under the same conditions as used for the xylobiase, showed that the activity of the xylanase decreased sharply as the DP was reduced (Fig. 2). The hydrolysis of xylobiose was extremely feeble, and could be detected chromatographically only after prolonged incubation. A slightly greater effect on xylotriose could be observed, small, roughly equal amounts of xylobiose and xylose being formed. Xylotetraose was much more readily hydrolysed; much xylobiose, and traces of xylotriose and xylose, appeared. The main products from xylopentaose were xylotriose and xylobiose, with a trace of xylose but no xylotetraose.

Xylohexaose yielded considerable amounts of xylotriose, with some xylobiose and xylotetraose and a trace of xylose, but no xylopentaose.

An unfractionated enzyme preparation from the *Butyrivibrio* sp. did not hydrolyse carboxymethylcellulose. The xylanase activity of the preparation was at least 100 times its 'cellulase' activity.

Arabinosidase activity. The electrophoretically more mobile xylanase fractions were able to remove arabinose from the wheat-flour pentosan and from the 'mixed oligosaccharides'; the less mobile fractions could not do so. The earlier of the fractions active in this respect hydrolysed xylobiose, but the later ones did not.

pH optimum and thermal stability. During incubation for 4 hr. in phosphate-citrate buffer solution, the activity against xylan had an optimum pH of 5.6 (Fig. 3). When heated at 60° at pH 6.5 about two-thirds of the activity against xylan was destroyed in 15 min., but some remained even after heating for 60 min.

DISCUSSION

The two strains of bacteria used in this work have many bacteriological similarities, and the similarity extends to their pentosan-decomposing enzymes. Although it has not been possible to compare the enzymes of the two strains in every particular, all the results which have been obtained suggest that these enzyme systems are identical. For the purposes of this discussion, no distinction will be drawn between results obtained by the use of enzymes from the two sources.

The behaviour of the enzyme preparations, both unfractionated and partially purified, towards the various substrates leads to the conclusion that the decomposition of pentosans by the two bacterial strains involves three enzymes: a xylanase, a β -xylosidase and an arabinosidase. The xylanase is the enzyme responsible for the initial breakdown of the xylan chain. It is an adaptive or inducible enzyme, produced only when the bacteria are growing in a medium containing xylan or a related polysaccharide. Under these conditions it is not the only pentosan-decomposing enzyme formed, and experiments with crude preparations do not indicate with certainty the properties of the enzyme itself. The small scale on which this work had to be done prevented us from preparing pure protein fractions by the conventional techniques. We have had some moderate success, however, in separating the different enzyme activities by zone electrophoresis, and we believe that the least-mobile fractions, although not necessarily containing only one protein, do contain only one of the enzymes involved in pentosan decomposition, namely the xylanase.

The mode of action of this enzyme shows it to be a typical endoenzyme, in that it splits more readily the xylosidic linkages near the middle of the chain. In so far as the xylanase attacks oligosaccharides, the same preference for inner linkages is found. Thus xylotetraose was split almost wholly at its central linkage to yield xylobiose. Xylopentaose was split mainly at one or other or both of the two inner linkages; attack at either of the two outer linkages was negligible, since no xylotetraose was detected during hydrolysis. Xylohexaose was similarly not attacked at the outer linkages; since the yield of xylotriose was considerably greater than the yield of xylotetraose plus xylobiose, the central linkage is presumably more accessible to the enzyme than are the two adjacent ones. The presence of arabinose units as side chains attached to the xylan appeared to inhibit the action of the xylanase. In the wheat-flour pentosan used in this work there was rather more than one arabinose to every two xylose units, i.e. if the side chains were uniformly distributed there would be a substituent on alternate xylose units. Xylose-xylose units protected in this way are apparently not accessible to the xylanase, and the observed partial decomposition of the pentosan is only possible because of uneven distribution of arabinose along the xylan chain (Perlin, 1951). The xylan used in these experiments, on the other hand, contained only one arabinose for every 12 xylose units, and so was readily attacked. Bishop & Whitaker (1955) observed that a purified cellulase from *Myrothecium verrucaria* hydrolysed the xylose-xylose linkages, but not the arabinose-xylose linkages, in a specimen of wheat-straw xylan. The converse effect, of a xylanase hydrolysing cellulose or its derivatives, does not seem to occur; the bacterial xylanase caused no detectable hydrolysis of carboxymethylcellulose.

The enzyme referred to above as 'xylobiase' is also inducible, but it is produced not only when xylobiose is the substrate, but also during growth on the polysaccharides. By cultivation of the bacteria on a medium containing xylobiose, the adaptive natures of the xylanase and xylobiase permitted the preparation of the latter freed from the former enzyme. This method yielded a purer specimen of xylobiase than could be obtained by electrophoresis. The enzyme is active against a wide range of xylose-containing oligosaccharides, upon each of which it acts by stepwise removal of single xylose units. The close parallel between the activities of the electrophoretic fractions against xylobiose and phenyl β -xyloside also suggests that this enzyme is a fairly non-specific β -xylosidase. It appears from Fig. 2 that the enzyme could attack oligosaccharides higher than the hexaose, and it is in fact able to release xylose from the 'mixed

oligosaccharides' but is without effect on the xylan.

The mode of action of the two enzymes separately suggests that the joint action of the xylanase and xylobiase on xylan is to cause a gradual fragmentation of the chain, leading finally to xylose as sole end product. This is exactly the pattern of action of the suspensions of mixed rumen bacteria (Howard, 1957) and of the unfractionated enzyme preparations from the two bacterial strains. Among the various oligosaccharides present during xylan hydrolysis, it is clear that no firm distinction can be made between those which are substrates of the xylanase only, and those which are substrates of the xylobiase only. There is considerable overlapping of the activities of the two enzymes; as the xylan chains become shorter they become less susceptible to the action of the xylanase and more susceptible to the action of the xylobiase.

Although purely hydrolytic in its action on dilute solutions of xylobiose, the xylobiase demonstrated a slight transferase activity when acting upon a concentrated solution. Other hydrolytic enzymes showing transferase activity under similar conditions, e.g. invertase (Allen & Bacon, 1956) and cellobiase (Crook & Stone, 1957), attach the majority of transferred glucosyl residues to primary alcohol groups in the receptor molecule. In the xylose-oligosaccharides there are, of course, no such groups, and probably for this reason the yield of synthetic oligosaccharides from xylobiose was very low. Crook & Stone (1957) observed that in transferring glucosyl residues to the secondary alcohol groups of cellobiose, a fungal cellobiase attached most to C-4 of the pyranose ring, less to C-3 and very little to C-2. A similar preference for that part of the molecule farthest from the glycosidic linkage was found in the bacterial xylobiase, leading to xylotriose as major product and 3²- β -xylosylxylobiose as minor product of transferase action on xylobiose.

The primary problem at present about the ability of the enzyme preparations to remove arabinose units from wheat-flour pentosan or the 'mixed oligosaccharides' is whether this is the work of a separate arabinosidase or is another aspect of the action of the xylanase or xylobiase. Conclusive proof of the existence of a separate arabinosidase would be afforded by the preparation of an active fraction free from xylanase and xylobiase activity. We have not yet encountered such material. We have, however, found arabinosidase activity in a xylobiase preparation free from xylanase, and also in some electrophoretically prepared xylanase solutions which were free from xylobiase. Furthermore, the arabinosidase activity of the xylobiase preparation seemed slightly more resistant to heat than did the xylobiase activity.

For these reasons we consider the arabinosidase to be a separate entity, not identical with either the xylanase or the xylobiase. This enzyme, like the other two, is inducible but in a curious way. It is not produced during growth on monosaccharides, but appears whenever either of the other two enzymes is formed. Hence it is produced during growth on xylobiose or rhodymenan, neither of which contains arabinose. Nothing further can be said at present about the properties of this enzyme, except that its electrophoretic mobility is intermediate between those of xylanase and xylobiase.

SUMMARY

1. Two strains of xylan-fermenting bacteria, one from the sheep and the other from the bovine rumen, have been grown in media containing a wide variety of xylans and related carbohydrates. Cell-free extracts have been prepared and tested for hydrolytic activity.

2. Two enzymes have been separated from the whole extracts by zone electrophoresis in starch columns.

3. The less mobile enzyme, referred to as xylanase, is formed by the two bacteria when these are grown on xylan or similar polysaccharide. It acts upon xylan by hydrolysing inner linkages, producing xylobiose as the main end product. It hydrolyses oligosaccharides similarly but has negligible action on xylotriose and xylobiose. Its effect on the xylan is inhibited by the presence of arabinose side chains.

4. The electrophoretically more mobile enzyme, referred to as xylobiase, is produced by the two bacteria when these are grown on xylose polysaccharides or xylobiose. It is a non-specific β -xylosidase, acting upon oligosaccharides by removal of single xylose units, and also hydrolyses phenyl β -xyloside. When acting upon a 20% (w/v)

solution of xylobiose, this enzyme synthesizes small amounts of two trisaccharides, xylotriose and 3²- β -xylosylxylobiose.

5. Some evidence is presented for the existence in the crude enzyme preparations of a discrete arabinosidase, which removes the arabinose side chains present in soluble pentosans. It appears to be formed by the bacteria whenever either xylanase or xylobiase is produced.

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APPENDIX

Isolation and Properties of the Xylan-Fermenting Bacterium 11

By JUANITA P. BUTTERWORTH, SHEILA E. BELL AND MARY G. GARVOCK
The Rowett Research Institute, Bucksburn, Aberdeen

(Received 18 June 1959)

The isolation and properties of the bacterium 11 (Institute collection) used in the preceding study of the degradation of xylans are described here.

METHODS

The medium used for isolation was the same as that described in the above paper for maintenance of stock

cultures, but without yeast extract and containing wheat-flour pentosan (0.25%). It was later found that better growth occurred with yeast added. Since growth occurred on this medium without added carbohydrate the medium used for determining the substrates utilized was altered in that the rumen fluid was replaced by Bacto-tryptone (0.2%) and Bacto-peptone (0.2%). In the presence of a suitable substrate this medium gave growth about equal to that of the rumen fluid medium. Very little growth