SOME PROPERTIES OF XYLANASE AND XYLOBIASE FROM MIXED RUMEN ORGANISMS

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Summary

Xylanase, free from xylobiase activity, has been obtained from crude extracts of mixed rumen organisms by fractionation on DEAE-cellulose. Xylobiase preparations were also obtained, but some xylanase activity was associated with all such fractions isolated.

Tris inhibits both xylanase and xylobiase activities, the latter, in most cases, being by far the more sensitive. Sodium, potassium, lithium, and ammonium ions are all effective in partially overcoming the inhibition due to Tris.

A number of compounds stereochemically related to Tris proved to be inhibitory to xylobiase, and an apparent relationship between structural properties and inhibitory activity is discussed.

Xylobiase-free xylanase hydrolyses xylotriose and higher xylo-oligosaccharides, but not xylobiose, maltose, cellobiose, melibiose, sucrose, lactose, cellulose, or starch. The xylobiase preparations isolated attack a wide variety of di- and trisaccharides.

There is no evidence that xylobiose is split by a phosphorolytic mechanism similar to that known for the cleavage of cellobiose.

I. INTRODUCTION

Investigations reported previously from this laboratory (Walker and Hopgood 1961) indicated that a purified hemicellulose-hydrolysing enzyme obtained from mixed rumen microorganisms had no detectable xylobiase activity. This led to the assumption that at least two enzymes are required for the degradation of xylose polymers to free xylose. Supporting evidence comes from the work of Howard, Jones, and Purdom (1960) who found that *Bacteroides amylogenes* and a *Butyrivibrio* species, both isolated from the rumen, produce separate enzymes for the hydrolysis of xylan to xylobiose and of xylobiose to xylose. Similarly, a crystalline xylanase purified by Inaoka and Soda (1956) from a *Bacillus* species produced xylose-oligosaccharides but not free xylose from xylan, indicating that this organism also contains a separate xylobiase.

Further studies on the xylanase and xylobiase activities of enzyme preparations from mixed rumen organisms have shown interesting inhibition characteristics and provided further evidence for the separation of the two enzyme activities into distinct entities.

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II. Materials and Methods

(*a*) *Reagents*

(i) *Xylan.*—This was a commercial product supplied by Nutritional Biochemicals Corporation.

(ii) *Xylobiose.*—Xylan (10 g) was dissolved as completely as possible with heating in 200 ml water. After cooling to room temperature penicillin (final concn. 30 units/ml), polymyxin B sulphate (final concn. 100 units/ml), and neomycin sulphate (final concn. 50 μg/ml) were added to prevent microbial contamination during the following step in the procedure. To this solution was added 200 ml of a dialysed aqueous extract of mixed, acetone-powdered rumen organisms (Walker and Hopgood 1961) and the pH adjusted to 6·2 with hydrochloric acid. The reaction mixture was then incubated at 37°C for 20 hr, at which time only xylose and xylobiose were detectable by paper chromatography. In order to remove protein, the mixture was heated at 100°C for 30 min, cooled, and filtered using Celite 545 as filter aid. The opalescent filtrate was then run through a column 4 cm in diameter by 28 cm long of a mixture of charcoal and Celite 545 (equal parts by weight) prepared according to Whistler and Tu (1952). Monosaccharides were removed from the column by eluting with 1500 ml of water and chromatographically pure xylobiose was recovered by elution with 5% ethanol, the first 700 ml containing all the recoverable xylobiose. The xylobiose was recovered as a syrup after concentration *in vacuo* and used without further treatment.

(iii) *Other Xylo-oligosaccharides.*—Xylotriose, xylotetraose, and xylopentaose were isolated from a partial enzymic hydrolysate of xylan by adsorption on a charcoal–Celite column as described in Section II(*a*)(ii). Elution was carried out with increasing concentrations of ethanol and the products finally obtained as syrups. Purity was checked by paper chromatography.

(iv) *Other Chemicals.*—All other chemicals used were commercial products of the highest purity obtainable.

(*b*) *Analytical Methods*

(i) *Assay of Xylanase Activity.*—Xylan was ground in a mortar with 0·1M potassium dihydrogen phosphate–disodium hydrogen phosphate buffer, pH 6·2, and the suspension diluted to give a final concentration of 25 mg/ml. To 0·2 of this suspension was added enzyme preparation and the volume adjusted to 0·4 ml. After incubation in stoppered tubes for 1 hr at 37°C Somogyi (1952) reagent was added and the reducing value determined after heating on a boiling water-bath for 30 min.

(ii) *Assay of Xylobiase Activity.*—The conditions for this assay were exactly as for xylanase with the exception that 0·1M xylobiose in phosphate buffer, pH 6·2, replaced xylan. In both cases, enzyme activity was calculated from the increase in reducing value over that of controls incubated without enzyme.

(iii) *Unit of Activity.*—One unit of activity for either xylanase or xylobiase was defined as "that amount of enzyme which is required to give an increase in reducing value of 0·001 ml of 0·005N copper under the described conditions of assay".

(iv) *Paper Chromatography.*—For separation of carbohydrate mixtures, a solvent system consisting of n-butanol–pyridine–water (6 : 4 : 3 v/v) was used. Sugars were located on the developed chromatogram with aniline phosphate reagent. In a search for sugar phosphates, paper chromatography was conducted using methanol–ammonia (sp. gr. 0·88)–water (16 : 1 : 3 v/v) as solvent (Bandurski and Axelrod 1951), phosphate-containing areas being located with the molybdate reagent of Hanes and Isherwood (1949).

TABLE 1

RATIO OF XYLANASE TO XYLOBIASE ACTIVITY IN VARIOUS PREPARATIONS FROM RUMEN ORGANISMS

For details of assay see Sections II(*b*)(i) and II(*b*)(ii)

Nature of Preparation	(A) Xylanase Activity (units/ml)	(B) Xylobiase Activity (units/ml)	Ratio (A/B)
Dialysed crude extract	$31 \cdot 0 \times 10^3$	$8 \cdot 5 \times 10^3$	3·6
Dialysed crude extract	$15 \cdot 0 \times 10^3$	$8 \cdot 0 \times 10^3$	1·9
60–80% acetone fraction from crude extract	$42 \cdot 9 \times 10^3$	$5 \cdot 2 \times 10^3$	8·2
Purified 84-fold with respect to xylanase	$17 \cdot 6 \times 10^3$	$4 \cdot 8 \times 10^3$	3·7
Purified 105-fold with respect to xylanase	$14 \cdot 5 \times 10^3$	$6 \cdot 9 \times 10^3$	2·1

III. RESULTS

(*a*) *Distribution of Xylobiase with respect to Xylanase*

Previously (Walker and Hopgood 1961) it was stated that hemicellulase purified 105-fold from rumen organisms had negligible activity towards xylobiose. Using the same enzyme preparation, it was subsequently observed that replacing the Tris–maleate buffer used at that time with phosphate buffer revealed xylobiose-hydrolysing activity. This finding led to an attempt to determine if both xylan and xylobiose were hydrolysed by the same enzyme(s). To this end, a number of crude extracts and fractions obtained during attempts to purify the two enzymes were assayed for their activities towards both xylan and xylobiose. Table 1 shows that the ratio of the two activities varied considerably in different preparations, although no complete separation had been achieved.

Further purification studies were undertaken following an observation that batchwise treatment of crude extracts of acetone-powdered rumen organisms with DEAE-cellulose removed xylobiase activity but left most of the xylanase activity in solution.

Crude extract of acetone-powdered rumen organisms containing 133,000 units of xylanase and 30,000 units of xylobiase activity and 12 mg of protein was applied to a column of DEAE-cellulose equilibrated with 0·005M dipotassium hydrogen phosphate–potassium dihydrogen phosphate buffer, pH 6·2. The dimensions of the column were 1·8 cm diameter by 6·5 cm long and the flow rate about 1 ml/min. The initial eluting buffer was 0·005M phosphate buffer, pH 6·2, 5-ml fractions being collected. Protein elution was followed by measuring optical density at 280 mμ and the concentration of phosphate in the eluting buffer was increased when protein elution reached a low value for a number of successive fractions. Figure 1 illustrates the pattern of elution of xylanase and xylobiase activities.

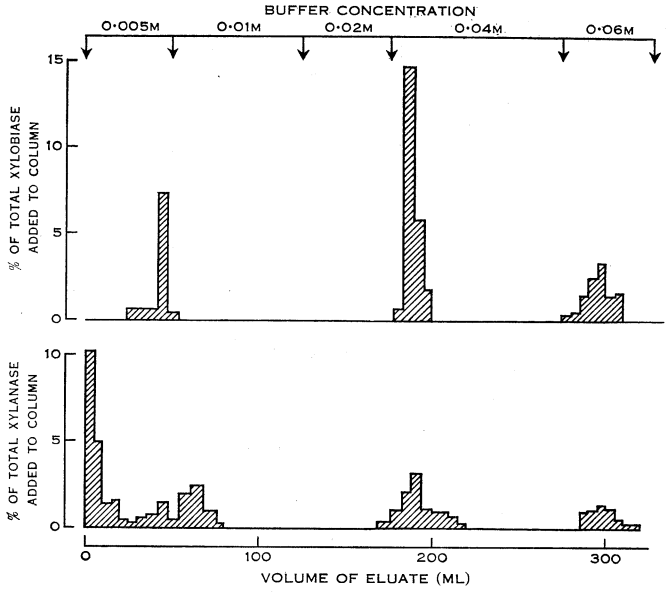


Fig. 1.—Elution of xylanase and xylobiase activities from a DEAE-cellulose column. Eluting buffer was dipotassium hydrogen phosphate–potassium dihydrogen phosphate, pH 6·2. Buffer concentration changed as shown.

In three peaks of activity, the elution of xylanase and xylobiase were very closely associated although the ratio of their activities was variable. However, the first six fractions eluted with 0·005M buffer contained 19% of the added xylanase activity without any associated xylobiase. A similar observation was made when a crude extract, previously treated with manganese chloride to remove nucleic acid (Kuby and Lardy 1953), was treated batchwise with DEAE-cellulose. In this case, in 0·005M phosphate buffer, the DEAE-cellulose adsorbed all the xylobiase activity leaving 60% of the xylanase in the supernatant. A further 34% of the xylanase together with 50% of the added xylobiase was recovered by eluting the DEAE-cellulose with 0·1M phosphate buffer, pH 6·2.

All attempts to separate xylobiase from xylanase by chromatography on DEAE-cellulose, solvent fractionation, and ammonium sulphate fractionation, failed.

(*b*) *Inhibition of Xylobiase Activity*

The observation that a purified xylanase preparation showing no activity towards xylobiose when assayed in Tris–maleate buffer (Walker and Hopgood 1961) did exhibit xylobiase activity in phosphate buffer was followed up by comparing the activity towards xylobiose in a range of different buffers all at pH 6·2 and 0·13M final concentration. It was found that xylobiase activity was the same in sodium–potassium phosphate, sodium arsenate–HCl, potassium hydrogen phthalate–NaOH, and sodium maleate–NaOH buffers but was greatly reduced in the presence of Tris–maleate buffer. In addition, replacement of part of the Tris–maleate by sodium–potassium phosphate partially relieved the inhibition. This experiment indicated that Tris was the inhibitory ion and that phosphate is not an essential reactant.

TABLE 2

EFFECT OF TRIS ON XYLOBIASE ACTIVITY

Enzyme and substrate incubated in sodium–potassium phosphate buffer and Tris (as the chloride). Final concentration of phosphate buffer 0·013M. Final concentrations of Tris as shown

Enzyme Preparation	Final Concn. of Tris (M)	Percentage Inhibition
Crude extract	0·0125	45
Dialysed crude extract	0·0125	37
Dialysed crude extract	0·0125	54
Dialysed crude extract	0·017	75
Dialysed crude extract	0·025	74
Purified 84-fold with respect to xylanase	0·0125	92
Purified 84-fold with respect to xylanase	0·017	100
Purified 105-fold with respect to xylanase*	0·017	72

* This assay contained additional sodium ion bringing the final concentration of alkali metal ions to 0·033M.

In the presence of 0·013M sodium–potassium phosphate buffer, pH 6·2, Tris (added as a solution adjusted to pH 6·2 with HCl) at concentrations below 0·01M did not significantly affect xylobiase activity. At concentrations of 0·013M and above, however, substantial inhibition of xylobiose breakdown was apparent. Table 2 summarizes the effect of Tris on a variety of enzyme preparations.

In order to investigate further the phenomenon of inhibition of xylobiase by Tris, a number of compounds stereochemically related to Tris and others containing hydroxyl or amino groups were examined for inhibitory capacity. The polyalcohols pentaerythritol, sorbitol, dulcitol, *meso*-inositol, ribitol, and glycerol were all ineffective as xylobiase inhibitors. However, compounds stereochemically related

to Tris were, to a varying extent effective. Table 3 shows that the active inhibitors were ethanolamine, 2-amino-2-methylpropane-1,3-diol, 3-aminopropanol, and 2-amino-2-methylpropan-1-ol in decreasing order of effectiveness. Ethanol and ethylamine were inactive.

TABLE 3

INHIBITION OF XYLOBIASE BY COMPOUNDS STEREOCHEMICALLY RELATED TO TRIS

Enzyme source was a dialysed crude extract of rumen organisms. All reaction mixtures contained 0·017M sodium–potassium phosphate, 0·017M xylobiose, and 0·017M inhibitor (final concentrations)

Inhibitor	Percentage Inhibition†
Ethanolamine	60
2-Amino-2-methylpropane-1,3-diol	36
2-Amino-2-hydroxymethylpropane-1,3-diol (i.e. Tris)	36
3-Aminopropanol	32
2-Amino-2-methylpropan-1-ol	24

† Activity in phosphate buffer without added inhibitor taken as maximal.

TABLE 4

EFFECT OF MONOVALENT CATIONS ON THE INHIBITION OF XYLOBIASE BY TRIS

Enzyme preparation was a crude dialysed extract of mixed rumen organisms. Tris added as the phosphate to a final concentration of 0·0125M. Cations added as the chlorides to a final concentration of 0·025M

Tris	Cation	Max. Observed Activity (%)
−	$Na^+ + K^+$	100
+	−	56
+	Na^+	69
+	K^+	69
+	Li^+	69
+	NH_4^+	75

Cohn and Monod (1951) and Kuby and Lardy (1953) showed that β-galactosidase is dependent for its activity on the nature of the cations present, the alkali-metal cations being required for maximal activity and substituted

ammonium ion being inhibitory. The effect of cations on the inhibition by Tris of xylobiase activity in a dialysed crude extract of rumen organisms was therefore studied. From Table 4 it is evident that sodium, potassium, and lithium were all equally effective in partially overcoming the inhibition by Tris and that ammonium ion was slightly more effective.

(c) *Differential Inhibition of Xylanase and Xylobiase by Tris*

Pursuing further the finding that in certain circumstances xylobiase activity may be completely inhibited by Tris, whereas xylanase is still active, a series of enzyme fractions were tested for their sensitivity to inhibition by Tris. Crude extracts and enzyme fractions purified by the method of Walker and Hopgood

TABLE 5

DIFFERENTIAL INHIBITION OF XYLANASE AND XYLOBIASE BY TRIS

Enzyme and substrate incubated in Tris phosphate buffer, pH 6·2

Enzyme Preparation	Tris Concn. (M)	Percentage Inhibition of Activity*	
		Xylobiase	Xylanase
Crude extract	0·025	100	22
Purified 84-fold with respect to xylanase	0·033	100	32
Purified 105-fold with respect to xylanase†	0·017	72	43
DEAE-cellulose fractions:			
1‡	0·025	—	0
9	0·025	23	0
35	0·025	17	22
56	0·025	88	41

* Activity in phosphate buffer taken as maximal.

† Also contained 0·02M sodium ion.

‡ This fraction contained no xylobiase activity.

(1961) showed far greater inhibition of xylobiase activity than of xylanase in the presence of Tris (Table 5). In these instances xylanase activity was always much higher than that of xylobiase. However, in one fraction obtained by chromatography on DEAE-cellulose in which xylobiase was more active than xylanase, the enzyme activities were equally sensitive to inhibition by Tris.

(d) *Substrate Specificity of the Enzyme Preparations*

The fractions obtained from DEAE-cellulose chromatography, although not characterized as homogeneous protein solutions, were examined for activity towards other carbohydrates.

The xylobiase-free xylanase not retained by DEAE-cellulose had no detectable activity towards maltose, cellobiose, melibiose, sucrose, lactose, cellulose, or starch. However, xylotriose, xylotetraose, xylopentaose, and xylohexaose were all hydrolysed. Fractions 35 and 36, which contained the bulk of the recovered xylobiase, exhibited a very wide range of activities. Cellobiose, maltose, melibiose, lactose, sucrose, melezitose, raffinose, and xylotriose all gave rise to enzymic hydrolysis products as determined by increase in reducing value. Confirmation was obtained using paper chromatography.

(e) *Search for Xylobiose Phosphorylase Activity*

Ruminococcus flavefaciens, a cellulose-digesting organism from the rumen, contains a cellobiose phosphorylase (Ayers 1959) which yields glucose and glucose-1-phosphate from cellobiose and inorganic phosphate. A small amount of maltose phosphorylase activity has also been detected in rumen organisms (Hobson and MacPherson 1952). Although inorganic phosphate was not essential for xylobiose hydrolysis by extracts of rumen organisms, it was possible that some xylobiose phosphorylase was present.

Chromatography of the products of xylobiose breakdown by a dialysed extract of mixed rumen organisms in the presence of phosphate buffer failed to reveal any phosphorylated carbohydrate. This lack of phosphate esterification was confirmed by using radioactive inorganic phosphate and isolating the barium-insoluble fractions as described by Trudinger (1956). Radioactivity determinations on the barium-insoluble fractions showed a complete absence of sugar phosphates.

IV. Discussion

The early studies on xylan hydrolysis by rumen organisms failed to resolve the question of the possibility of the presence of two enzymes, one degrading long-chain anhydroxylose polymers to xylobiose and the other hydrolysing xylobiose to free xylose. Sørenson (1955) postulated the presence of two separate enzyme activities, but Pazur *et al.* (1957), on the basis of their studies, concluded that a separate xylobiase was unlikely. Howard, Jones, and Purdom (1960) resolved the issue by demonstrating that both *Bacteriodes amylogenes* and a *Butyrivibrio* species produced separate enzymes for the hydrolysis of xylan and xylobiose when grown on pentosan substrates.

The work reported here to some extent confirms the findings of Howard, Jones, and Purdom (1960) with the demonstration that a xylobiase-free xylanase can be isolated from mixed rumen organisms. There is also a degree of agreement with their findings in that xylobiase activity could not be completely separated from xylanase, since they were obliged to grow their organisms on xylobiose in order to obtain a xylobiase preparation "free" from xylanase activity and even such preparations had a trace of activity towards pentosan. In the rumen, of course, the organisms are obliged to utilize the fodder pentosans, so that in view of the findings of Howard, Jones, and Purdom, it may be expected that xylanase activity would to a small extent accompany xylobiase activity. This in fact is the case when fractionation on DEAE-cellulose is applied to crude extracts.

Further evidence for the existence of two enzymes, or in some cases perhaps two active centres on the same enzyme, for the hydrolysis of xylan and xylobiose arises from the differential activity of Tris in the inhibition of enzyme activity. In all cases but one, xylobiase activity is much more sensitive to inhibition by Tris than is xylanase. The exception is the fraction from chromatography on DEAE-cellulose containing the bulk of recovered xylobiase. It seems possible that, since the level of inhibition is much the same for both enzyme activities, this fraction contains a rather non-specific xylobiase which is associated with a small amount of xylanase activity, similar to the xylobiases isolated by Howard, Jones, and Purdom (1960).

The range of sugars hydrolysed by the xylobiase-free xylanase and the xylobiase-containing fractions from DEAE-cellulose chromatography differs enormously. As was found in a previous xylanase purification study (Walker and Hopgood 1961) activity of xylanase towards carbohydrates other than those composed of xylose units is undetectable. In its ability to hydrolyse oligosaccharides of the xylose series, the xylanase studied here resembles that investigated by Howard, Jones, and Purdom (1960).

The very wide range of sugars hydrolysed by the major xylobiase-containing fraction from DEAE-cellulose may indicate that xylobiase as a specific enzyme does not exist in the rumen, and that xylobiose is split by non-specific β-glycosidases. The other possibility is that this enzyme fraction was heavily contaminated with other glycosidases, and since maltose and sucrose are hydrolysed, there is certainly some α-glucosidase and invertase present. The latter explanation may be the more likely in view of the fact that the xylobiases studied by Howard, Jones, and Purdom (1960) has no activity towards cellobiose, a 1,4-β-glucoside.

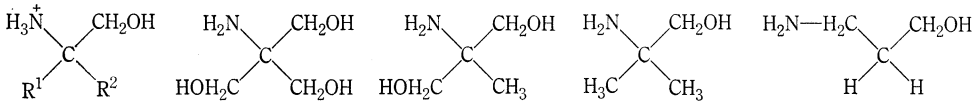
The complete failure to demonstrate the formation of phosphate esters during xylobiose breakdown by crude extracts of mixed rumen organisms indicates the absence of any mechanism resembling the phosphorolytic cleavage of cellobiose described by Ayers (1959).

One of the most interesting findings from the present study is the inhibition of enzyme activity by Tris and related compounds. A similar finding was reported by Kuby and Lardy (1953) who observed an almost complete inhibition of the β-galactosidase of *Escherichia coli* in the presence of 0·14M Tris and absence of other cations, and a 49% inhibition with 0·072M Tris plus 0·068M sodium ion. These findings are similar to those reported in this paper (Tables 2 and 5).

The ability of alkali metal ions to partially overcome the inhibition of xylobiase activity by Tris parallels the findings of Kuby and Lardy (1953) and Cohn and Monod (1951) who observed a requirement by *E. coli* β-galactosidase for monovalent cations, the most active of which were sodium and potassium.

Among the hydroxyamino-compounds stereochemically related to Tris, there seems to be an association between inhibitory activity and structural properties. Compounds with hydroxyl groups only (sugar alcohols and ethanol) and with an amino group only (ethylamine) are non-inhibitory. The common feature of the inhibitory compounds is the possession of a primary alcohol and an amino group,

and at pH 6·2 the inhibitory ion would have the structure (I). The substituents at R^1 and R^2 appear to influence the degree of inhibition obtained. Ethanolamine, the most powerful inhibitor of the series studied, has the simplest possible substituents at R^1 and R^2. In Tris (II) and 2-amino-2-methylpropane-1,3-diol (III) extra



(I) (II) (III) (IV) (V)

primary alcohol groups are introduced, and, bearing in mind the tetrahedral arrangement of the substituent groups about the central carbon atom, one might expect an inhibitory potential greater than for ethanolamine because of the increased probability of an amino and primary alcohol group of the correct conformation being presented to the enzyme. The fact that Tris and 2-amino-2-methylpropane-1,3-diol are less effective than ethanolamine may be due to crowding of the space near to the amino group by the fairly large substituent groups at R^1 and R^2. Thus, we have the "probability" effect increasing inhibitory potential, but being overcome by crowding of the amino group. The least effective inhibitor of the series, 2-amino-2-methylpropan-1-ol (IV) has fairly large substituent groups at R^1 and R^2 and has no advantage of the "probability" effect. Finally, it seems that the spatial separation of the alcohol and amino groups may also be a determinant of inhibitory activity since 3-aminopropanol (V) is only half as effective as ethanolamine although in both compounds the substituents at R^1 and R^2 are hydrogen.

V. Acknowledgments

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