days at 30°. The intermediate produced yielded 2.3 g. of purified sapogenin. This was an over-all yield of 23% of the sapogenin which should have been obtained from this sample by using the regular procedure.

The remainder of the extract, containing saponins extracted from 1 kg. leaves, was adjusted with concd. HCl to *p*H 1.5 and refluxed 4 hours. From the resulting partial hydrolysis solids 6.2 g. of sapogenin was recovered, or 107% of the yield by the regular procedure. The data are given in Table II.

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Enzymatic Hydrolysis of Xylan[1]

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An enzyme preparation from the medium of *Aspergillus foetidus* hydrolyzed corn cob xylan primarily to xylobiose with no production of D-xylose. An extract of the mold mycelium was separated on a cation-exchange resin into two fractions; one which hydrolyzed xylan and xyloöligosaccharides to D-xylose without producing significant amounts of transient oligosaccharides and one which hydrolyzed xylan to a mixture of D-xylose and xyloöligosaccharides. Fractionation of the mycelium extract on either celite or carboxymethylcellulose produced a fraction which did not hydrolyze xylan but did hydrolyze hemicellulose-B.

Xylan is hydrolyzed by animals,[2] plants,[3] and by a large number of microörganisms.[4] Hydrolysis of xylan by microörganisms was probably first examined by Hoppe-Seyler.[5] Sorenson[6] recently gave evidence that certain bacteria act on xylan to produce xyloöligosaccharides and D-xylose.

Since the use of specific xylan splitting enzymes would be of great assistance in examining the structure of the hemicelluloses, this Laboratory has undertaken the separation of the enzymes of *Aspergillus foetidus* into preparations with specific hydrolytic actions. Herein is described the initial separations achieved.

An extract of *A. foetidus* mycelium hydrolyzes xylan to D-xylose and xyloöligosaccharides as indicated by chromatographic comparison of the products with authentic samples.[7] This enzyme preparation can be separated on a cation exchange resin into two fractions. A fraction (I) splits xylan to D-xylose, cellulose to D-glucose and hydrolyzes hemicellulose-B. Fraction II splits xylan to D-xylose and xyloöligosaccharides, cellulose to D-glucose and cellobiose but does not hydrolyze hemicellulose-B. A further fractionation of fraction I on a column of carboxymethylcellulose yields a portion (IA) that like fraction I hydrolyzes xylan, cellulose and hemicellulose-B and a portion (IB) that does not hydrolyze xylan and cellulose but does hydrolyze hemicellulose-B.

The mold mycelium extract can also be separated on columns of either celite or carboxymethylcellulose into a fraction of unchanged activity which hydrolyzes xylan, cellulose and hemicellulose-B, and another fraction which hydrolyzes hemicellulose-B but does not hydrolyze xylan or cellulose.

Fractional precipitation of the medium with ammonium sulfate yields a preparation which hydrolyzes xylan to xylobiose and higher xyloöligosaccharides but produces no noticeable D-xylose. This "xylo-dextrinase" has no effect on cellulose.

It is recognized that chromatographic comparisons do not constitute absolute identifications of D-xylose and xyloöligosaccharides. However such rapid and simple chromatographic comparisons with authentic specimens provide strong indications of the components present. This is particularly true in examining the enzymatic hydrolysis products of xylan because the structure of the polysaccharide is fairly well established and because its acid hydrolytic products have been well characterized as crystalline substances.[7]

Experimental

Materials.—Xylan was prepared from corn cob as previously described.[8] Uronic acid: 2.9%, $[\alpha]^{25}D$ −116.3° (*c* 1.0 in 1 *N* NaOH); ash, 0.45%; molecular weight by light scattering, 34,500; D.P., 261. Hemicellulose-B was also prepared as previously described. Swollen or "reactive" cellulose was prepared according to the phosphoric acid procedures outlined by Walseth.[9,10] The mold, *A. foetidus*[11] was

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grown on a liquid medium of 1.5% D-glucose, 3.0% bactopeptone and inorganic salts.[12]

Identification of Oligosaccharides.—Paper chromatographic analyses of the hydrolyzates of xylan by the various enzyme preparations were compared to authentic xylooligosaccharides previously isolated and characterized.[7] Descending chromatograms were developed at 25° on Whatman No. 1 paper with a basic solvent of ethyl acetate, pyridine and water in a ratio of 10:4:3 and with an acidic solvent of ethyl acetate, acetic acid, formic acid and water in a ratio of 18:3:1:4. The papers were dried and sprayed with *p*-anisidine hydrochloride.[13] Colored areas resulting from interaction of spray reagent and sugars appeared after heating in an oven for 1 minute at 120°. The oligosaccharides in the enzyme hydrolyzate correspond in R_x values (ratio of distance traveled to the distance D-xylose travels in the same time) with authentic crystalline xyloöligosaccharides.[7] R_x values of known xyloöligosaccharides in the above-mentioned acidic and basic developing agents are given in Table I.

TABLE I

R_x VALUES OF XYLOÖLIGOSACCHARIDES

Sugars	Eluant: Acidic	Eluant: Basic
Xylobiose	0.380–0.400	0.678–0.690
Xylotriose	.145–0.152	.427–0.440
Xylotetraose	.038–0.050	.236–0.242
Xylopentaose	.0013	.110
Xylohexaose		.0395
Xyloheptaose		.0285
Xyloöctaose		.0157

Enzyme Fractionation.—Two liters of autoclaved medium was inoculated with mold spores. After four days at 25°, before the mold had begun to form spores, the medium was drained from the mycelium, suction filtered through Whatman No. 57 paper and 80% saturated with ammonium sulfate. After 12 hours, the precipitate was removed by centrifugation and dialyzed at 4° in a collodion sac against frequently changed distilled water for 4 days. The resulting enzyme solution was freeze-dried to yield approximately 0.92 g. of a tan colored powder. Its nitrogen content of 9.6% indicated 60.1% protein present.

Thirty mg. of this enzyme preparation was added to 75 mg. of xylan in 15 ml. of *p*H 4.0 buffer solution at 37°. During the hydrolysis, aliquots were regularly taken for chromatographic analysis. In none of these was there any evidence of D-xylose; xylobiose appeared to be the smallest carbohydrate molecule present. The hydrolysis was slow, taking 24 hours to degrade 9.25% of the substrate. The reducing values, calculated as D-xylose, were used to determine the extent of hydrolysis.[14] The enzyme had no noticeable action on cellulose. However, an enzyme preparation obtained in a similar manner from the medium of a spored 7-day old mold hydrolyzed xylan to D-xylose and xyloöligosaccharides. This change in activity may be accounted for by autolysis of cells which might release a greater amount of enzymes into the medium at this stage.

The mycelium of a 4-day old *A. foetidus* culture was ground in a blendor with fine clean sand and a sufficient amount of 5% aqueous sodium chloride solution to produce a thick slurry. The cell-free extract was filtered through Whatman No. 1 paper on a buchner funnel and the residue washed with fresh 5% sodium chloride solution. The filtrate was 80% saturated with ammonium sulfate and allowed to stand at 4° for 12 hours. The precipitate was centrifuged and dialyzed at 4° in a colloidon sac against distilled water for 4 days. Freeze-drying produced a tan powder which hydrolyzed xylan to D-xylose and xyloöligosaccharides. The nitrogen content of the powder was 9.1% indicating a protein content of 56.8%.

A column (2 × 13 cm.) of 140 mesh IRC-50 resin in the hydrogen form was washed with MacIlvaine's citric acid–phosphate buffer at *p*H 5.8.[15] After addition of 0.6 g. of the dried cell-free mycelium extract, the column was eluted with *p*H 6.5 buffer at 20° at the rate of 1 ml. per 3 minutes. One-ml. fractions were collected and added to 1.0 g. of dry ashless xylan or hemicellulose-B in 11 ml. of *p*H 4.5 buffer. The hydrolysis proceeded at 37° for 48 hours. After this period the reducing values (as D-xylose) of the substrates were determined and chromatograms made. Chromatograms of the xylan hydrolyzates produced by the first 12 to 22 ml. of eluate, fraction I, showed the production of D-xylose only, while all other active eluates II produced D-xylose and other xyloöligosaccharides. A plot of the reducing values for xylan against the ml. of eluant gave a bimodal curve, with the major apex corresponding to the hydrolytic action of fraction I and the minor apex corresponding to the hydrolytic action of fraction II. Chromatographic evidence and reducing values indicated that only fraction I hydrolyzed hemicellulose-B. Fraction I also hydrolyzed cellulose to D-glucose while fraction II hydrolyzed cellulose to D-glucose and cellobiose.

The separation was repeated on additional cell-free extract. The fractions with D-xylose producing activity were combined and freeze-dried to give 0.30 g. of a tan powder (I). This enzyme preparation contained 9.8% nitrogen indicating 61.3% protein.

Fraction I was separated on a 1 × 13 cm. column of carboxymethylcellulose[16] (degree of substitution, 0.563) previously washed with *p*H 5.1 mono and dibasic sodium phosphate buffer. Three hundred mg. of fraction I was dissolved in 3 ml. of water, placed on the column and eluted with *p*H 6.5 phosphate buffer. The elution was done as rapidly as possible as the enzyme preparation can hydrolyze carboxymethylcellulose.[17] Two fractions were separated. One fraction (IA) found in the first 11 ml. of eluate retained the original activity of I and hydrolyzed xylan to D-xylose and hemicellulose-B to D-xylose and various oligosaccharides. A second fraction (IB) eluted after the first 11 ml. hydrolyzed hemicellulose-B to oligosaccharides with no evidence of D-xylose. Xylan is not affected by this fraction.

Optimum *p*H of Dried Enzyme Preparations.—Thirty mg. individual samples of the mycelium extract and of the fraction I and 60-mg. samples of the medium extract were each added to 50-mg. individual samples of xylan in 5 ml. of water. Five-ml. aliquots of six different MacIlvaine buffers were added to each sample at 37°. After 5 hours reducing values were determined. By assuming completion of the hydrolysis when all the xylan was hydrolyzed to D-xylose, the ratio of the reducing value to the final reducing value was taken as a measure of the extent of hydrolysis.

TABLE II

EFFECT OF *p*H ON ENZYMATIC ACTIVITY

Source of enzyme preparation	*p*H	Hydrolysis, %
Medium	2.8	14.8
	3.0	15.8
	3.4	17.2
	3.6	16.6
	4.0	15.2
	5.0	12.2
Mycelium	3.0	13.2
	4.0	16.4
	4.5	18.4
	5.0	16.0
	5.5	13.0
Fraction I	3.0	17.8
	3.6	19.7
	3.8	20.4
	4.0	19.8
	4.5	18.4
	5.0	17.2

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The medium extract gave an optimum hydrolysis at *p*H 3.4, the mycelium extract at *p*H 4.5 and the fraction I at *p*H 3.8 (Table II).

Optimum Temperature.—Samples of enzyme preparations were weighed out similar to those used previously to determine the optimum *p*H. Ten ml. of *p*H 3.4, 3.8 and 4.5 buffers were added to the medium extract, the fraction I and the mycelium extract, respectively. These enzyme solutions were individually added to 50 mg. of xylan. Mixtures of each enzyme preparation were held at 25, 37, 45 or 55° for 5 hours. At this period, reducing values determined the optimum temperature for the mycelium and medium extracts as 37° and the fraction I as 45° (Table III).

TABLE III

EFFECT OF TEMPERATURE ON ENZYMATIC ACTIVITY

Source of enzyme preparation	Temp., °C.	Hydrolysis, %
Medium	25	8.0
	37	17.2
	45	15.2
	55	Nil
Mycelium	25	12.0
	37	18.8
	45	16.4
	55	9.7
Fraction I	25	22.0
	37	30.8
	45	32.2
	55	28.2

Heat Inactivation.—Solutions containing 100 mg. of dried cell-free mycelium extract preparation or 100 mg. of dried medium extract preparation in 5 ml. of water were heated at 65°. At intervals 0.5 ml. of the solution was added to 50-mg. samples of xylan in 2 ml. of *p*H 4.0 buffer at 37°. After standing for 20 hours chromatograms were made to determine whether hydrolysis had occurred. Inactivation of the medium extract occurred after 5 minutes heating. The mycelium extract that had been heated for less than 30 minutes hydrolyzed xylan to D-xylose and the xyloöligosaccharides as indicated by paper chromatography. On the other hand the mycelium extract that had been heated for longer than 30 minutes hydrolyzed xylan to D-xylose only. Fraction I is not inactivated after 6 hours heating and thus by heating the mycelium extract at 65° for 30 minutes the fraction I activity can be separated from the fraction II activity.

Enzyme Action on Oligosaccharides.—Individual samples of 25 mg. of pure xylobiose, xylotriose and xylotetraose were each dissolved in 2 ml. of water. To one series 2 ml. of *p*H 3.4 buffer and 25 mg. of dried medium extract were added. To another series 10 mg. of dried fraction I and 2 ml. of *p*H 3.8 buffer were added. Both series were maintained at 37°. The course of hydrolysis was followed by taking hourly aliquots of 10 λ (microliters) for chromatographic analysis.

Search for Phosphorylated Sugars.—Phosphorylated sugars may be detected on a paper chromatogram by a variety of color tests. One of the most satisfactory procedures is Gutzeit's method as modified by Whistler and Smart.[10] This method did not indicate the presence of phosphorylated sugars either as intermediates or end products in the hydrolysis of xylan or in the hydrolysis of the xyloöligosaccharides by any of the enzymes described.

TABLE IV

ENZYME ACTION ON XYLOÖLIGOSACCHARIDES

Enzyme preparation	Oligosaccharide substrate	End product(s)	Total time elapsed, hr.
Medium extract	Xylobiose	No reaction	..
	Xylotriose	No reaction	..
	Xylotetraose	Xylobiose	18
Fraction IA	Xylobiose	D-Xylose	8
	Xylotriose	Xylobiose and D-xylose	4
		D-Xylose	8
	Xylotetraose	Xylotriose, xylobiose and D-xylose	4
		Xylobiose and D-xylose	7
		D-Xylose	8

Separation of Xylan-splitting from Hemicellulose-B-splitting Enzymes.—The enzymes responsible for the hydrolysis of hemicellulose-B were separated from the enzymes responsible for xylan hydrolysis by two methods.

The first method was a modification of Van Sumere's.[18] Three grams of powdered mycelium extract was dissolved in 75 ml. of water 66% saturated with ammonium sulfate. This solution was mixed with 15 g. of celite, placed in a glass tube, 2 cm. in diameter, and allowed to settle at 20°. The column was eluted with 33% saturated ammonium sulfate solution at a rate of 5 ml./15 minutes. Each 5 ml. of effluent was added to 13 ml. of *p*H 4.5 solutions of xylan or cellulose or hemicellulose-B at 37° (60° for the cellulose mixture). After 24 hours the reducing values (calculated as D-xylose for xylan and hemicellulose-B and D-glucose for cellulose) were determined. The fraction III eluted in the first 115 ml. was active against xylan, cellulose and hemicellulose-B. The fraction IV eluted after this point was active against hemicellulose-B only.

A second method of enzyme separation involved the use of carboxymethylcellulose prepared as described above. Using a 300-mg. sample of mycelium extract on a *p*H 5.1 buffered column and eluting with *p*H 6.5 buffer, produced a fraction III in the first 9 ml. which was active against xylan, cellulose and hemicellulose-B. Upon further elution a second fraction IV was separated which hydrolyzed only hemicellulose-B.

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