[CONTRIBUTION OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

The Polysaccharide Associated with Yeast Invertase

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The polysaccharide associated with purified invertase has been shown to be identical with the mannan, yeast gum, obtained by autolysis of yeast. The methylated yeast invertase polysaccharide gave upon hydrolysis 2,3,4,6-tetra-*O*-methyl-D-mannose (2 moles), 2,4,6-tri-*O*-methyl-D-mannose (1 mole), 3,4,6-tri-*O*-methyl-D-mannose (1 mole) and 3,4-di-*O*-methyl-D-mannose (2 moles).

Previous investigations into the composition of yeast invertase have indicated that purified preparations contain varying amounts of carbohydrate.[2-7] The carbohydrate was not identified, but since it gave no precipitate with Fehling solution it was generally assumed to be distinct from the yeast gum[8,9] which accompanies invertase in yeast autolysates. The enzyme was thought to be a protein–carbohydrate complex.[12]

During the course of this work it was observed that the test for yeast gum with Fehling solution[10] is less sensitive in the presence of proteins. The possibility arose, therefore, that the carbohydrate associated with yeast invertase may have been yeast gum which could not be detected because of the presence of relatively large amounts of protein. This proved to be the case.

The carbohydrate polymer present in purified invertase preparations was isolated by several methods (see Experimental section). One method, utilizing charcoal, enabled the carbohydrate to be separated from the enzyme without destroying the activity of the latter. In all cases the isolated carbohydrate polymer was shown to be identical with yeast mannan (yeast gum). Both had the same specific rotation and the corresponding acetates had the same rotation. Both showed the same precipitation reaction with Fehling solution and with the Jackbean globulin, concanavalin-A.[2,10,11]

Acid hydrolysis of the carbohydrate associated with invertase proceeded slowly and yielded only D-mannose; yeast gum behaved in the same way. Moreover, both the yeast invertase polysaccharide and yeast mannan consumed the same amount of periodate (1.1 moles per mole of anhydromannose) with the liberation of the same amount of formic acid (0.3 mole).

Finally methylation studies established the identity of the two polysaccharides. The two methylated polysaccharides were indistinguishable; they had the same rotation and both furnished upon hydrolysis the same cleavage products, namely, 2,3,4,6-tetra-*O*-methyl-D-mannose (2 moles), 2,4,6-tri-*O*-methyl-D-mannose (1 mole), 3,4,6-tri-*O*-methyl-D-mannose (1 mole) and 3,4-di-*O*-methyl-D-mannose (2 moles).

A linear polysaccharide composed of D-mannose units joined by 1,3-glycosidic bonds was not encountered in this work.[12]

Experimental

Isolation of Mannan from Baker's Yeast. (1) By Alkaline Extraction of Yeast.—This is essentially the procedure used by Haworth, Hirst and Isherwood[8] which has been modified as follows: yeast was autoclaved at 12 lb. pressure for 2 hours with 6% NaOH and the mannan in the filtrate precipitated with 1 volume of ethanol and purified by preparation of the copper complex using alkaline Benedict solution. After dissolving in 2 *N* HCl, ethanol (1 vol.) was added to effect precipitation. The procedure was repeated and the product was washed with acetone to remove an acetone-soluble copper compound which appears not to be removed by alcohol precipitation alone (yield 14–15 g. per kg. yeast). The mannan was finally freed of the last traces of copper by passage over a cation-exchange resin IR 120. Precipitation of the eluate with 3–4 volumes of methanol gave a colorless product with $[\alpha]^{25}D$ +89° in water (*c* 1.0) which yielded only mannose on hydrolysis.

(2) From Yeast Autolysates.—Yeast autolysates were prepared as described by Sumner and O'Kane[2] or Adams, Richtmyer and Hudson.[3] The autolysates were treated with calcium phosphate gel[2] or bentonite[3] as described by the above authors for the adsorption of invertase and the supernatant solutions were made slightly alkaline with dilute sodium hydroxide. Benedict solution was then added with stirring until all the mannan was precipitated. Purification was effected by the procedure described above (yield 14–15 g. per kg. yeast). The colorless product showed a specific rotation of $[\alpha]^{25}D$ +89° in water (*c* 1.0) and was composed entirely of mannose as revealed by hydrolysis and paper partition chromatographic analysis.

Isolation of the Polysaccharide Present in Invertase. (1) By Ethanolic Fractionation of Alkali-treated Invertase.—Invertase (400 mg.) prepared by the Sumner and O'Kane method[2] was dissolved in 25 ml. of 1 *N* NaOH. After 10 minutes the solution was acidified slightly with 1.5 *N* acetic acid and then treated with 1 volume of ethanol. The precipitate was centrifuged and washed with ethanol followed by ether. This fraction 1 weighed 125 mg. and had $[\alpha]^{25}D$ +80° in water (*c* 1.0). Fraction 2, weighing 85 mg., having $[\alpha]^{24}D$ +42° in water (*c* 1.0) was obtained by the addition of another volume of ethanol to the supernatant liquid after removal of fraction 1. The addition of a third volume of ethanol to the supernatant liquid from fraction 2 gave 150 mg. of fraction 3, $[\alpha]^{24}D$ +6° in water (*c* 1.5).

The ultraviolet absorption data indicated that fraction 1 contained little if any protein since it displayed no selective absorption in the ultraviolet region of the spectrum whereas the other two fractions, showing strong selective absorption, appeared to contain relatively large amounts of protein.

(2) By Use of Carbon.—A column (25 × 120 mm.) consisting of equal parts by weight of Darco G60 and Celite was used for the adsorption of invertase (20 mg. in 0.5 ml. of water). After washing the column with 100 ml. of water,

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20% ethanol (300 ml.) was passed through. All of the invertase carbohydrate was eluted by this treatment at room temperature. No invertase activity was present in the eluate whereas the carbon column was noted to have approximately the same activity as was present prior to the elution procedure. Heating of the alcohol-eluted carbon–Celite column with 2 N H_2SO_4 for 2 hours gave a hydrolysate in which no sugar could be detected by chromatographic analysis.

The progress of carbohydrate elution was followed by means of a Molisch test carried out as follows: To 4 drops (0.2 ml.) of eluate and 1 drop (0.02 ml.) of 1% α-naphthol in 95% ethanol was added 1 ml. of concentrated sulfuric acid and the contents mixed. The presence of mannan was indicated by the development of a purple color. It was possible to detect 5 μg. of carbohydrate by this test.

The eluate was also examined for the presence of yeast mannan by means of the concanavalin-A reagent[13] or by the use of Benedict solution as follows. One ml. of eluate was treated with 0.3 ml. of Benedict solution and 0.3 ml. of 15% NaOH. The mixture was shaken thoroughly and allowed to stand. Fifty μg. of mannan may be detected as a precipitate in less than a minute and 10 μg. may be detected after 10 or 15 minutes if the mixture is shaken occasionally.

Under the above conditions a sample of yeast mannan behaved in the same way in that it was eluted with 15–20% ethanol.

(3) **By Treatment with 2,4-Dinitrofluorobenzene.**—A solution of purified invertase (50 mg. in 2 ml. of water) was treated with 3 ml. of a 5% alcoholic solution of 2,4-dinitrofluorobenzene and 1 ml. of 5% sodium bicarbonate added. The mixture was shaken for 3 hours and then extracted three times with 3 ml. of ether. The supernatant liquid was decanted and the yellowish dinitrophenyl derivative washed twice with 2 ml. of 1 N sodium hydroxide. The supernatant liquid and washings were combined and the polysaccharide precipitated by means of Benedict solution. Further purification was effected as described above.

Periodate Oxidation of the Yeast Polysaccharides.—Treatment of yeast mannan or of the mannan obtained from purified invertase with 1.5 moles of potassium metaperiodate showed identical consumption of 1.1 moles of periodate per mole of anhydromannose in 3 days at room temperature. No formaldehyde could be detected by means of the dimedon test, but both reaction mixtures showed the formation of 0.30–0.32 mole of formic acid per anhydromannose unit.

The mannan polyaldehyde was reduced with sodium borohydride[14] and the polyalcohol so formed subjected to hydrolysis and analysis by paper chromatographic methods.[15] The products formed were mannose and glycerol; no erythritol was detected.

Fischer and Kohtes[12] obtained a mannose polysaccharide from a yeast autolysate which had been treated with chloroform and 1-butanol to remove protein. Their mannan, which contained a small amount of nitrogen, was sparingly soluble in water; it consumed only a small amount of periodate which was taken to indicate that the polysaccharide was linear and composed of 1,3-linked mannose units. These results were of considerable interest since they indicated the presence of a new polysaccharide in yeast. Although a 1,3-linked polysaccharide has been previously isolated from the yeast cell wall,[16] it differs from that described by Fischer and Kohtes in containing only glucose and in being insoluble even in dilute alkali.

In contrast to the results obtained by Fischer and Kohtes,[12] we found that after treatment of yeast autolysate with chloroform and 1-butanol, the isolated polysaccharide behaved like yeast mannan in being precipitated by concanavalin-A or by alkaline Benedict solution. It was soluble in water and, moreover, it consumed approximately 1 mole of periodate per anhydro mannose unit in agreement with the behavior of both yeast mannan and the invertase polysaccharide. The polysaccharide contained approximately 1% nitrogen and upon hydrolysis with dilute sulfuric acid it yielded only mannose.

It is believed that the results obtained by Fischer and Kohtes might have been due to the use of a preparation containing denatured protein which rendered the polysaccharide insoluble in water and resistant to attack by periodate. Some support for this view was obtained by noting that when a yeast mannan fraction obtained from yeast autolysate and containing about 2% nitrogen was brought into contact with hydrogen chloride in a desiccator, it gave a product which was practically insoluble in water and which consumed little periodate. However, dilute alkali dissolved the mannan-denatured protein complex and the resulting solution then gave the characteristic reactions of yeast mannan.

Hydrolysis and Composition of the Enzyme Polysaccharide and of Yeast Mannan.—Hydrolysis was accomplished by heating each of the polysaccharides (20 mg.) in a sealed tube with 2 N H_2SO_4 (0.5 ml.) for 5 hours in a boiling water-bath. These conditions were found to give maximum recovery (85–90%) of reducing sugars as determined by the dinitrosalicylic acid method of Sumner and Howell.[17] Neutralization of the hydrolysate with barium carbonate, filtration and concentration *in vacuo* gave a sirup which on a paper chromatogram, developed with butanol-1:ethanol:water (5:1:4), showed the presence of mannose and small amounts of slow-moving components (probably mannose oligosaccharides). The chromatograms were sprayed with aniline oxalate and heated for 15 minutes at 110°. No monosaccharide other than mannose was detected. This was demonstrated chromatographically using several solvents and by preparation of mannose phenylhydrazone, m.p. and mixed m.p. 197°.

The mannose was separated from the slow-moving material present in the hydrolysate by means of a charcoal–celite column[18] and eluted with water. Thereafter 10% ethanol was passed through the column. No further amount of carbohydrate could be eluted by increasing the concentration of ethanol. The 10% aqueous alcoholic eluate was evaporated *in vacuo* and the sirup was placed on a paper chromatogram which was developed with *t*-amyl alcohol:1-propanol:water (5:1:2). It was found that four spots, all traveling slower than mannose, could be detected. When these slow moving components were separated and hydrolyzed further with 2 N H_2SO_4 for 2 hours they furnished only mannose.

These relatively stable mannose oligosaccharides may eventually prove to be useful for confirming the structure of yeast mannan.

Methylation of the Polysaccharide (Yeast Mannan) Associated with Yeast Invertase.—The polysaccharide associated with invertase was methylated *via* the acetate ($[\alpha]^{24}D$ +64° in chloroform (c 1.0)), according to the procedure described by Haworth, Hirst and Isherwood.[8] The methylated polysaccharide showed a specific rotation of $[\alpha]^{20}D$ +88° in chloroform (c 1.0). Found: OMe, 43.7%.

Hydrolysis of the Methylated Polysaccharide and Separation of the Methylated Sugars.—The methylated polysaccharide (1.0 g.) was hydrolyzed with a mixture of equal volumes of glacial acetic acid (10 ml.) and 5% hydrochloric acid (10 ml.).[8] After neutralizing the hydrochloric acid with barium carbonate and evaporating to dryness *in vacuo*, the methylated sugars were extracted with chloroform (yield 1.07 g.).[19] For separation of the sugars an automatic fraction collector was used in conjunction with a hydrocellulose–cellulose column (4 × 130 mm.)[20] and the solvent, methyl ethyl ketone:water azeotrope.[21] One gram of the hydrolysate was completely separated by one pass through the column into three fractions. Thus, using a collection time of 12 minutes per tube, the tetra-*O*-methyl fraction (0.355 g.) was present in tubes 28–45, the tri-*O*-methyl fraction (0.320 g.) in tubes 47–72 and the di-*O*-methyl fraction (0.310 g.) in tubes 103–128. The recovery from the column was 98.5% and the ratios of the methyl-

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(19) When acetone was used for the extraction much of the di-*O*-methyl fraction was converted to an acetone derivative which moved with the tetramethyl fraction on paper chromatograms or on the hydrocellulose column (developed with methyl ethyl ketone:water azeotrope).

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ated fractions were 1.00:0.96:0.99 for the tetra:tri:di-*O*-methyl-D-mannose fractions, respectively. The methylated fractions were identified by paper chromatographic analysis using the descending technique with methyl ethyl ketone:water as developing solvent. Aniline oxalate[22] was used to locate the methylated sugars on the chromatogram.

Characterization of the Methylated Components. (a) **2,3,4,6-Tetra-*O*-methyl-D-mannose.**—The fast moving component did not crystallize but treatment with aniline in the usual way gave the anilide of 2,3,4,6-tetra-*O*-methyl-D-mannose, m.p. and mixed m.p. 143°, $[\alpha]^{24}D$ −5° equilibrium value in methanol (*c* 2.0) (after recrystallization from ether–petroleum ether). Values reported[9] for the anilide of 2,3,4,6-tetra-*O*-methyl-D-mannose are: m.p. 144° and $[\alpha]D$ −7.5° in methanol.

Anal. Calcd. for $C_{16}H_{25}O_5N$: C, 61.7; H, 8.1. Found: C, 61.8; H, 7.7.

(b) **3,4,6-Tri-*O*-methyl-D-mannose.**—The middle fraction (tubes 47–72), 0.32 g., separated from the hydrocellulose column, was dissolved in dry acetone (20 ml.) and sulfuric acid (0.15 ml.) added. After standing 24 hours, the sulfuric acid was removed as ammonium sulfate by precipitation with dry ammonia gas and the filtered solution evaporated *in vacuo*. Extraction of the residual sirup with several small portions of petroleum ether gave a liquid which failed to crystallize. The sirup was treated with 0.05 *N* sulfuric acid and heated on the water-bath for 2 hours. After removing the acid with barium carbonate, filtering and evaporating the filtrate *in vacuo*, a sirup was obtained which crystallized on standing overnight. Recrystallization from ether or ether–light petroleum ether gave 3,4,6-tri-*O*-methyl-D-mannose (0.045 g.), m.p. and mixed m.p. 102°, $[\alpha]^{23}D$ +28° initial value in water (*c* 1.0). A melting point of 102° and $[\alpha]D$ +27.5° initial value in water are reported for 3,4,6-trimethylmannose.[9]

In other experiments the crystalline 3,4,6-tri-*O*-methyl-D-mannose was also obtained directly without passing through the acetone derivative by dissolving the sirupy trimethyl fraction in a small amount of ether and concentrating to a small volume and adding a nucleus. Crystallization was complete after standing overnight. The supernatant liquid was removed and the crystals washed three times with small amounts of an ether–petroleum ether mixture (2:1). The crystalline substance thus obtained had m.p. and mixed m.p. 102°.

The 3,4,6-tri-*O*-methyl-D-mannose consumed approximately 1 molecular proportion of potassium periodate in 24 hours at room temperature in the dark (3.1 mg. consumed 1.01 ml. of 0.0131 *M* KIO_4).

Anal. Calcd. for $C_9H_{18}O_6$: C, 48.6; H, 8.2. Found: C, 48.8; H, 8.6.

(c) **2,4,6-Tri-*O*-methyl-D-mannose.**—It was noted that on the paper chromatogram the 3,4,6-tri-*O*-methyl derivative became brown after spraying with aniline oxalate and heating whereas the residue which remained from the trimethyl fraction after removal of the greater part of the crystalline 3,4,6-tri-*O*-methyl derivative gave a tan color with aniline oxalate.

After treatment of the trimethyl fraction with periodate for several days, the test for formaldehyde by the use of chromotropic acid[23] was negative. It therefore appears that 2,3,4-tri-*O*-methyl-D-mannose cannot be present in more than trace amounts.[9]

Treatment of a portion of the whole trimethyl fraction obtained directly from the column with potassium metaperiodate in the usual way showed the consumption in 24 hours of 0.52 mole of periodate per mole of tri-*O*-methyl sugar. The periodate-oxidized material was evaporated to small volume and treated with 2 volumes of ethanol. After filtration, the filtrate was concentrated to a small volume and placed on a strip of Whatman No. 3 filter paper. When the chromatogram was developed with methyl ethyl ketone:water, two components were located. One traveled at a rate faster than 2,3,4,6-tetra-*O*-methyl-D-mannose and produced a violet gray color on spraying with aniline oxalate while the other band traveled at a rate similar to 3,4,6-trimethylmannose, but gave a tan color with aniline oxalate. The R_f value of this tri-*O*-methyl derivative and the color it gave with aniline oxalate corresponded to those of 2,4,6-tri-*O*-methyl-D-mannose. The fast moving component traveled at the same rate as 2,3,5-tri-*O*-methyl-L-arabinose which likewise gave a violet-grey color on treatment with aniline oxalate. It was presumably 2,3,5-tri-*O*-methyl-D-arabinose and arose by cleavage of C_1 from the 3,4,6-tri-*O*-methyl-D-mannose.

The slow moving band was eluted from the strip with 50% ethanol and treated with potassium metaperiodate. Essentially no periodate was consumed in 48 hours indicating that this tri-*O*-methyl-D-mannose is the 2,4,6-tri-*O*-methyl derivative. The R_f value of 2,3,6-tri-*O*-methyl-D-mannose was different (0.67) from that (0.58) of the two trimethylmannose derivatives encountered here, using methyl ethyl ketone–water azeotrope as the irrigating solvent.

(d) **3,4-Di-*O*-methyl-D-mannose.**—The slowest moving fraction (tubes 103–128 (0.31 g.) which crystallized directly upon removal of the solvent had the same R_f value as authentic 3,4-di-*O*-methyl-D-mannose on paper chromatograms using methyl ethyl ketone–water azeotrope or 1-butanol–ethanol–water (5:1:4). After recrystallization from acetone–ether it separated as the monohydrate, m.p. 113°, $[\alpha]^{20}D$ +3° equilibrium value in water (*c* 3.0). 3,4-Di-*O*-methyl-D-mannose is reported[9] to have m.p. 114° and $[\alpha]D$ +4° in water.

Anal. Calcd. for $C_8H_{18}O_7$: C, 42.6; H, 8.0. Found: C, 42.5; H, 8.2.

When a sample of yeast gum obtained from autolyzates as described above, was methylated, hydrolyzed and carried through the same analytical procedures as described above, the results paralleled those for the enzyme polysaccharide.

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