[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY]

Enzymatic Hydrolysis of Guaran[1,2]

BY ROY L. WHISTLER, W. H. EOFF[3] AND D. M. DOTY[4]

Introduction

Guaran is the principal polysaccharide of guar seed endosperm. Its structure, based on physical measurements of its triacetate films,[5,6] on periodate oxidation,[7] on methylation data,[8,9] and on other chemical information, seems to be that of a chain of 1,4′ linked D-mannopyranose units, one-half of which have a single D-galactopyranosyl unit substituted on the hydroxyl group at carbon atom C6. On the basis of X-ray patterns[10] obtained from the stretched polysaccharide films, it has been concluded that a mannan chain with side chains of one galactose unit on every second mannose unit is in best accord with the data.

Further information on the detailed structure of guaran can be obtained by examination of the oligosaccharide fragments produced when the molecule is enzymatically hydrolyzed. Heyne and Whistler[5] observe that a commercial diastase preparation causes a reduction in the viscosity of guaran solution. The present work was undertaken to prepare an enzyme capable of rapid hydrolysis of guaran. Some of the products of the enzyme action have been described.[11]

Experimental

Malt Diastase Extract.—Approximately 4 g. of malt diastase (Merck) was extracted with 100 ml. of distilled water for thirty minutes at room temperature, the mixture centrifuged and the clear centrifugate separated.

Cephalosporium Extract.—A species of the mold *Cephalosporium* was isolated when found growing as a chance contaminant on an exposed guaran solution. To about one liter of Czapek's (acid) solution containing 1% of dissolved guar flour was added 1 ml. of an actively growing *Cephalosporium* mold culture (growing in guaran solution). After five days at 28° the material was filtered by means of a Seitz filter and the filtrate reserved.

Guar Seed Extract.—About 1 kg. of guar seeds (Guar seeds were kindly supplied by Mr. D. C. Aepli, Arizona Experiment Station, Mesa, Arizona) were treated with 75% sulfuric acid for thirty minutes at room temperature and then washed free of acid. The seeds were allowed to germinate between moist blotters for two days at 28°. They were alternately frozen and thawed three times to break up the tissue structure, violently agitated in an equal volume of water in a Waring blendor for about one minute and then slowly stirred at room temperature for six hours. Centrifugation removed all solid material and left a clear, slightly amber colored extract.

A comparison of the enzyme activities present in the three extracts was made by following the viscosity decrease which they produce on a 1% solution of guaran. The guaran was prepared according to the procedure of Heyne and Whistler.[5] An amount to produce a 0.5% solution was dispersed in water by means of a Waring blendor and the dispersion autoclaved at 15 lb. steam pressure for 15 minutes. Five ml. of the malt extract, 1 ml. of *Cephalosporium* extract and 1 ml. of germinated guar seed extract were added to separate 100-ml. portions of 0.5% guaran solution at 37° and the change in viscosity observed. The results indicate that by far the greatest activity is to be found in the extract of germinated guar seeds. Consequently, this material was employed as a source of enzyme for most of the further investigations.

Although malt diastase causes a decrease in the viscosity of a solution of guaran, experiments indicate that the active enzyme is neither *alpha*- nor *beta*-amylase but is due to the presence of another enzyme similar in properties to *beta*-amylase. Thus while a preparation of malt *beta*-amylase obtained by inactivation of the *alpha* enzyme attacked guaran, a sample of crystalline *beta*-amylase obtained from Dr. A. K. Balls of the Western Regional Research Laboratory at Albany, California, did not lower the viscosity or increase the reducing power of a guaran solution.

Enzyme Purification.—Ammonium sulfate fractionation was used to concentrate the enzyme activity from germinated guar seeds and to bring about partial purification. The extract from germinated guar seeds was saturated with ammonium sulfate. The precipitate which contained practically all of the guaran-hydrolyzing activity was dissolved in distilled water and fractionally precipitated by addition of increasing amounts of ammonium sulfate.

Activities of the various fractions were tested by measurements of reducing sugars formed when aliquots were allowed to act on guaran solutions. Thirty-five ml. of 0.715% guaran solution sufficient to give a final concentration of 0.5% and 10 ml. of a citric acid–disodium phosphate buffer solution[12] at *p*H 4.8, were added to a 50 ml. volumetric flask and the flask placed in a constant temperature bath at 40°. When temperature constancy was obtained there was added 5 ml. of solution containing 1 mg. of enzyme preparation per ml. The reducing value of the solution was determined at various time intervals by removing an aliquot and estimating reducing sugars by the method of Blish and Sandstedt[13] as modified for 0.1 *N* reagents by Sandstedt.[14] Most of the activity was concentrated in the 20–30% ammonium sulfate precipitated fraction. The 10–20% ammonium sulfate fraction exhibited about two-thirds as much activity while other fractions evidenced only small activities.

Isolation of Dry Enzyme.—Dialysis was shown to cause no loss in enzyme activity. To obtain quantities of dry enzyme a procedure was adopted in which the 20–30% ammonium sulfate precipitate was dialyzed against distilled water until free of sulfate ion. The solution was then centrifuged to remove small amounts of precipitate and the clear centrifugate lyophilized. The activity per unit weight, based on its ability to increase the reducing power of a guaran solution at *p*H 4.8 and 40°, was ap-

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(2) Paper presented before the Division of Sugar Chemistry at the 116th meeting of the American Chemical Society at Atlantic City, N. J., September, 1949.

(3) Barley and Malt Laboratory, Madison, Wisconsin.

(4) American Meat Institute Foundation, Chicago, Illinois.

(5) E. Heyne and R. L. Whistler, THIS JOURNAL, **70**, 2249 (1948).

(6) C. L. Smart and R. L. Whistler, *J. Polymer Sci.*, **4**, 87 (1949).

(7) R. L. Whistler, T. K. Li and W. Dvonch, THIS JOURNAL, **70**, 3144 (1948).

(8) J. Swanson, *ibid.*, **71**, 1510 (1949).

(9) Z. F. Ahmed and R. L. Whistler, *ibid.*, **72**, 2524 (1950).

(10) K. J. Palmer and M. Ballantyne, *ibid.*, **72**, 736 (1950).

(11) D. F. Durso and R. L. Whistler, paper presented before the Division of Sugar Chemistry at the 117th meeting of the American Chemical Society at Detroit, April, 1950.

(12) H. T. S. Britton "Hydrogen Ions," Chapman and Hall, Ltd., London, 1929, p. 187.

(13) M. J. Blish and R. M. Sandstedt, *Cereal Chem.*, **10**, 189 (1933).

(14) R. M. Sandstedt, *ibid.*, **14**, 604 (1937).

proximately four times greater than that of the enzyme before dialysis.

Attempts to further purify the enzyme by fractional precipitation from water solution with ethanol were unsuccessful. The fractions were far less active than the starting enzyme, indicating that the enzyme was partially inactivated by the ethanol treatment.

Optimum *p*H.—Based on measurement of reducing power the optimum *p*H of the dialyzed and lyophilized enzyme at 40° on a 0.5% solution of guaran was 5.0 for a citric acid–disodium phosphate buffer and was *p*H 4.4 for a sodium acetate–acetic acid buffer.

Optimum Temperature.—For these measurements 1 mg. of enzyme was added for each 10 ml. of a 0.5% solution of guaran which was buffered at *p*H 5.0 with citric acid–disodium phosphate. The optimum temperature as indicated by measurement of reducing power was 45° for periods of time up to one hour, 40° for periods of about one to two and one-half hours and 35° for longer periods. At 35° no appreciable inactivation of the enzyme occurred.

Kinetics and Substrate Concentration.—At 35°, guaran concentration of 0.5% and an enzyme concentration of 1 mg. per 10 ml. of solution, a constant number of reducing sugar equivalents are liberated per unit of time through the test period of four hours. Thus at this substrate concentration the reaction rate is of zero order. At guaran concentrations in the neighborhood of 0.1% or less the reaction rate is no longer of zero order.

Inhibitory Effect of Ammonium Sulfate.—To 100 ml. portions of guaran solutions containing 0.00, 0.01, 0.10% and 1.0% ammonium sulfate were added equal amounts (1 ml.) of dialyzed and lyophilized enzyme (1 mg. per ml.). By following the increase in reducing power it was observed that ammonium sulfate gave no inhibitory effect until a concentration of greater than 0.1% was reached.

Extent of Guaran Hydrolysis.—A 0.5% solution of guaran at 35° and *p*H 5 was hydrolyzed by enzyme during a period of thirty-six hours to a reducing sugar value of 80% of that for complete hydrolysis. This extent of hydrolysis was not increased by further addition of enzyme. Partial analysis of the hydrolysate showed that 65% of the guaran had been converted to monosaccharides while the remainder consisted principally of di- and tri-saccharides.

Discussion

Although the enzyme preparations from guar seeds increase the reducing value and decrease the viscosity of guaran solutions, it is observed that the maxima of these two effects do not coincide for all enzyme preparations. Thus, it is likely that two or more guaran-hydrolyzing enzymes are present. The occurrence of at least two enzymes is probable since the enzyme preparation brings about not only hydrolysis of mannosido bonds in the main chain but also the hydrolysis of the galactosido bonds.

Regardless of whether the enzyme preparation is a mixture it is valuable as a tool for hydrolyzing guaran to produce oligosaccharide fragments, the identification and characterization of which will lead to further information regarding the structure and properties of guaran. The enzyme may also be of value for the commercial modification of industrial galactomannans.

Summary

An enzyme preparation separated from germinated guar seeds has been found to cause a guaran solution to undergo both a rapid decrease in the viscosity and a rapid increase in reducing power. As characterized by its ability to increase the reducing power of a guaran solution the enzyme preparation shows greatest activity at *p*H 5 in a citric acid–disodium phosphate buffer and at *p*H 4.4 in an acetic acid–sodium acetate buffer. The effect of temperature on the enzyme action is also examined. It is likely that more than one enzyme is present. While malt diastase causes a decrease in viscosity of a guaran solution the results suggest that the active enzyme is neither alpha- nor beta-amylase but an enzyme impurity similar in properties to beta-amylase.

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Structures of the OXO Aldehydes from Styrene

BY LEWIS A. WETZEL, CHARLES H. MCKEEVER AND CHARLES L. LEVESQUE

In two recent publications, Adkins and Krsek[1] have reported the preparation of 2-phenylpropionaldehyde in 30% yield by the application of the OXO reaction to styrene. The aldehyde was identified by its semicarbazone. There was no evidence of the simultaneous formation of 3-phenylpropionaldehyde, which, from the results obtained with alkenes,[2] would be expected to be a major product. Adkins and Krsek noted this discrepancy and mentioned that the 3-phenylpropionaldehyde was probably formed, but not isolated. When this work was repeated in these Laboratories, again the semicarbazone of 2-phenylpropionaldehyde was the only one readily isolated. There was, however, evidence that another semicarbazone was present.

We have obtained additional evidence that the primary product of the OXO reaction on styrene is actually a mixture of the 2- and 3-phenylpropionaldehydes. The data indicate that at least 23% is the 2-isomer, at least 29% is the 3-isomer, while the remaining 47% cannot be assigned to one or the other at present. Following Adkins' procedure,[1] but limiting the time of distillation, styrene gives a 46% yield of phenylpropionaldehydes. Oxidation of this aldehyde mixture with gaseous oxygen gives 3-phenylpropionic acid and acetophenone in approximately equal amounts. The former must come from the oxidation product of 2-phenylpropionaldehyde, via the following steps

(1) Adkins and Krsek, (a) THIS JOURNAL, **70**, 383 (1948); (b) **71**, 3051 (1949).

(2) *E. g.*, Keulemans, Kwantes and van Bavel, *Rec. trav. chim.*, **67**, 298–308 (1948).