over 0.00008% iron, and at all times this reagent should be tested with the 1% potassium iodide solution for the presence of oxidizable foreign substance, assumed to be oxidized iron.

Under these conditions the above mentioned difficulties do not arise and the method of analysis can be carried through with the accuracy mentioned in the former publication.[5]

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Carbohydrate-Fatty Acid Linkings in Corn Alpha Amylose[1]

BY T. C. TAYLOR AND RUTH T. SHERMAN

The fatty acids associated with corn starch occur entirely in the α-amylose[2] or insoluble portion of the starch, while the soluble β-amylose is pure carbohydrate. Thus the presence of the fatty acids affords one means of differentiating between the two corn amyloses. In the interests of obtaining more data on chemical make-up of each of the corn amyloses, an examination of the linkage between the fatty acid groups and the carbohydrates in corn α-amylose is desirable. This investigation is concerned with that problem.

It has been shown that the fatty acid compounds in corn α-amylose are derived from palmitic, oleic and linolic acids,[3] which are chemically combined with carbohydrate for they are not extracted by solvents, but are liberated only after relatively long aqueous acid hydrolysis of the amylose.

In spite of the fact that these acids are in chemical combination with the carbohydrate, no corn α-amylose has been prepared which contains all that is in the original starch. Some is always lost in the process of separating the α-amylose from the β-amylose. This applies also to the derivatives of starch. Acetylated and methylated products of corn starch containing fatty acids have been prepared,[4] but in neither case do the products contain more than half of the original acids. This suggests that the fatty acids may not all be linked to the carbohydrate molecule in the same manner.

As starch is a polyhydroxylated compound, the fatty acids can be combined to the carbohydrate through an oxygen linking at any one or more of the otherwise free hydroxyl groups of the glucose residues which form the amylose molecule.

(1) An abstract of a dissertation presented by Ruth T. Sherman to the Faculty of Pure Science of Columbia University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) (a) Taylor and Nelson, THIS JOURNAL, **42**, 1726 (1920); (b) Taylor and Iddles, *Ind. Eng. Chem.*, **18**, 713 (1926).

(3) Lehrman, "The Fatty Acids in Corn Starch and Synthesis of Corn Beta Amylose Palmitate," Columbia Dissertation, 1925.

(4) Werntz, "Studies of the Corn Amyloses," Columbia Dissertation, 1926.

The most direct and satisfactory way of determining the position of the fatty acids on the carbohydrate molecule would be to hydrolyze α-amylose to a glucose unit with the fatty acid radicals still attached and investigate the fragment. This cannot be accomplished at present, as the hydrolyzing agent splits off some fatty acids in the very early stages of hydrolysis of the starch.

Another method by which some light might be shed on the structure of corn α-amylose is to study the ease of removal of the fatty acids by hydrolysis. If the fatty acid residues are not all attached at the same relative positions on the glucose residues in the α-amylose, the stability of the starch–fatty acid linkages toward hydrolytic reagents ought not to be the same. Some linkages may be expected to be more easily broken than others, according to the methods of hydrolysis employed, if one uses the behavior under similar conditions of derivatives of glucose with substituents in various positions as a criterion. Here, in general, a substituent at position 1 in the glucose molecule is found to be the easiest to split off.[5]

The immediate purpose of this paper is to report the results of the effect of acid, alkali and enzymic hydrolytic reagents on corn α-amylose.

I. **The Effect of Acid on the Liberation of the Fatty Acids from Corn Starch.**—First, the preferential removal of these acid residues by a mild hydrolyzing agent, hydrogen chloride in 95% alcohol,[6] was studied. Samples of corn starch were treated with the reagent for different lengths of time, and the fatty acid content of the carbohydrate *residue* was determined.

After a given treatment the residue was freed from extraneous fatty acids and then completely hydrolyzed. Any newly liberated fatty acids were extracted and their iodine number determined. When this iodine value is compared with the iodine number of the fatty acids liberated by complete hydrolysis of the original starch, it is possible to determine the order in which the fatty acid groups are split off in the partial hydrolyses. If the iodine number of the acid in the residue increases, the palmitic acid must have been liberated more rapidly; if it decreases, the unsaturated acid linkings must have been hydrolyzed more readily than that of the palmitic acid.

Experimental

Five hundred cc. of a solution of 0.12 g. of hydrochloric acid in 95% alcohol was added to a suspension of 500 g. of corn starch in 500 cc. of alcohol. Samples of this mixture were heated under a reflux condenser on a water-bath for twenty minutes, forty minutes and one hour, respectively, after which they were filtered while hot on a suction filter and extracted for eight hours with ether. A weighed part of the treated

(5) (a) Armstrong, *J. Chem. Soc.*, **85**, 1048 (1904); (b) Will and Lenze, *Ber.*, **31**, 68 (1898); (c) Fischer, *ibid.*, **49**, 584 (1916).

(6) By the use of alcoholic acid the amount of reducing sugar usually formed when aqueous acid is used is greatly diminished. The alcohol also cuts down the tendency of the granules to swell and disrupt.

starch in each case and a sample of untreated but ether-extracted starch was completely hydrolyzed with aqueous acid according to the method of Taylor and Nelson[2a] and the liberated fatty acids washed, dried, extracted, weighed and their iodine number found.

The iodine number was determined by the pyridine–dibromide method of Rosenmund and Kuhnhenn,[7] except that after adding the pyridine–dibromide solution, the mixture was kept in the dark and shaken frequently, and that the excess of iodine was titrated with 0.05 *N* sodium thiosulfate solution.

The results are given in Table I. It can be seen that as the amount of the still-combined fatty acid in the treated starch decreases, its iodine number decreases. This shows that on treatment of corn starch with a mineral acid, the unsaturated acid radicals, oleyl and linolyl, are split more readily from the amylose than the saturated palmityl radical. The latter remains with the amylose and is only liberated when the amylose is more completely hydrolyzed.

TABLE I

THE EFFECT OF ALCOHOLIC HYDROGEN CHLORIDE ON THE FATTY ACID CONTENT OF CORN STARCH

Time of treatment	Fatty acid content of residue,[a] %	Iodine numbers	Av.
None	0.73	93.03, 93.09, 93.01, 92.66, 92.28, 92.51, 92.96	92.78
Twenty minutes	.44	73.07, 72.56, 73.36, 73.11, 72.78	72.97
Forty minutes	.21	53.64, 53.83, 53.71, 53.72	53.72
One hour	.059	27.51, 28.84, 26.75	27.37

[a] Average of two analyses.

II. **The Effect of Basic Reagent on the Liberation of the Fatty Acids from Corn Starch.**—In alkaline solutions, unmodified starch granules swell and form viscous pastes, but they still give a characteristic blue color with iodine after acidification. However, the fatty acid content of corn starch is greatly decreased by certain alkaline treatments. Rask and Phelps[8] report that heating corn starch for a short time with alcoholic ammonium hydroxide removes the fatty acids quantitatively, although in this Laboratory it has been shown that eight successive treatments with this reagent are needed before the fatty acid content of the starch becomes negligible.[9]

This reagent was used therefore in studying the action of alkaline reagents on the removal of the fatty acids from corn starch. In Table II will be found the results.

III. **The Effect of Amylases on the Liberation of Fatty Acids.**—The fate of the fatty acids in corn starch after treatment with an amylase has been reported in only one instance. Taylor and Nelson[2a] have found that on allowing corn starch to react with malt amylase, most of the fatty acids were liberated during the early stages of hydrolysis, but the amylase

(7) Rosenmund and Kuhnhenn, *Z. Untersuch. Lebensm.*, **46**, 154 (1923).
(8) Rask and Phelps, *Ind. Eng. Chem.*, **17**, 187 (1925).
(9) Taylor and Werntz, THIS JOURNAL, **49**, 1584 (1927).

TABLE II

THE EFFECT OF ALCOHOLIC AMMONIUM HYDROXIDE ON THE FATTY ACID CONTENT OF CORN STARCH

Number of treatments	Fatty acid content of residue,[a] %	Iodine numbers	Av.
None	0.75	93.03, 93.09, 93.01, 92.66, 92.28, 92.51, 92.96	92.78
One Sample I	.28	60.60, 60.67, 60.88, 60.83	60.74
One Sample II	.27	59.54, 59.71, 59.88, 59.67	59.70
Two	.18	46.13, 42.24, 46.23, 46.13	46.18
Three	.13	36.87, 36.90, 36.98, 36.90	36.91

[a] Average of two analyses.

preparation used by them had not been tested for fat-splitting enzymes. Any lipase present might be expected to hydrolyze the fatty acid compounds, irrespective of the action of the amylase. This will be demonstrated below.

In this work the action of a substantially lipase-free amylase on corn starch was investigated in order to ascertain its hydrolytic effect on the combined fatty acids. Should such an amylase attack the fatty acid bearing portion of the corn starch (the α-amylose), it could either split the carbohydrate, leaving the fatty acids combined with some product of lower molecular weight, or it could attack the carbohydrate–fatty acid linkages or both.

Corn starch was treated with several commercial amylases of different lipase content, in order to determine whether the presence of lipase will affect the quantity of fatty acid liberated during the course of the hydrolysis. The two most likely amylases were "Taka" diastase and "Superase."[10]

The lipase content of the enzyme was estimated by allowing the amylase to react on triolein and titrating the ether-extracted free oleic acid formed after the well-shaken samples had stood for twenty-four hours. The "Taka" diastase mixture was buffered at a P_H of 4.52 and held at 30° while the "Superase" was buffered at a P_H of 7.3 and held at 60°.

One sample of "Superase" liberated no oleic acid and another liberated about 0.5% while the "Taka" diastase liberated 1.5% of the acid. The first "Superase" sample is apparently substantially free from lipase.

Both the "Superase" and the "Taka" diastase were allowed to react with corn starch and the effect on the liberation of fatty acids studied. The results are given in Table III.

Experimental

Twenty-gram samples of starch were mixed with 100 cc. of cold distilled water and poured into 700 cc. of boiling water, taking care that no lumps were formed. To those samples containing the Taka diastase, 100 cc. of acetate buffer of P_H 4.52 was added; to those containing "Superase," 100 cc. of phosphate buffer of P_H 7.2–7.3. The samples were well stirred and kept in an oven for twenty-four hours. For those containing

(10) (a) Furnished by Wallerstein Laboratories, 171 Madison Avenue, New York City. (b) Furnished by the Takamine Laboratory, Inc., Clifton, New Jersey, under the trade name "Clarase C."

"Taka" diastase the temperature was kept at 30°, and for those with "Superase," at 60°. They were then acidified with acetic acid until acid to methyl red, and a small quantity of mercuric chloride was added to each as a preservative. They were filtered through wet filter papers and washed with distilled water; the filter papers were dried overnight in an oven at 40°, and extracted for three days in a Soxhlet apparatus with ether and the residue in the flask weighed after evaporation of the solvent.

TABLE III

THE AMOUNT OF FATTY ACID HYDROLYZED FROM CORN STARCH BY THE AMYLASES

Amylase per 20 g. starch, g.	Fatty acid split by Taka diastase, g.	% of Total fatty acids split by Taka diastase	Fatty acid split by "Superase," g.	% of Total fatty acids in starch split by "Superase"
0.050	0.0162	12.2	0.0057	4.3
.100	.0244	18.4	.0075	5.6
.200	.0451	33.9	.0103	7.7

Fatty acid content of the starch, 0.73%. Time of treatment, 24 hrs.

From the results recorded in Table IV it is evident that the amylase containing lipase ("Taka" diastase) hydrolyzes the greater quantity of fatty acid from corn starch. The "Superase," which showed a practically negligible amount of lipase in the test on olein, also liberates the smallest amount of fatty acids from the corn starch.

When "Superase" is used directly on the corn α-amylose instead of the starch containing α-amylose there are similar results as shown in Table IV.

TABLE IV

THE REMOVAL OF THE FATTY ACID FROM α-AMYLOSE BY AN AMYLASE

Grams of amylase per 3 g. of α-amylose	Grams of fatty acid split from 3 g. of α-amylose		% Total fatty acid split	
0.050	0.0054	0.0048	4.5	4.0
.100	0.0060	0.0057	5.0	4.8
.200	0.0050	0.0056	4.2	4.7
.400	0.0078	0.0079	6.6	6.6
.600	0.0077	0.0083	6.5	6.9
1.000	0.0125	0.0127	10.7	10.7

The fatty acid content of the α-amylose was 3.8%.

Turning to the production of reducing sugar during this treatment, it was found in two preliminary experiments that when using 0.05 g. of "Superase" per 20 g. of starch made as a 2% paste, the maximum amount of reducing sugar occurred after twenty-four hours. For example, when several 50-cc. aliquots of the mixture were titrated iodimetrically by the Willstätter–Schudel[11] method the reducing sugar expressed in milligrams of iodine per 500 mg. of starch (50-cc. aliquots contain 500 mg. of dry starch) was 170 and 171 for a twenty-four hour treatment and 169 and 170 for a forty-eight hour treatment.

By increasing the amount of amylase that acts on a given amount of

(11) (a) Willstätter and Schudel, *Ber.*, **51**, 780 (1918); (b) Goebel, *J. Biol. Chem.*, **72**, 801 (1927).

starch, the production of reducing sugar increases to a maximum for that concentration also at about twenty-four hours for optimum conditions.

That the degree of dispersion plays no essential part is shown by the fact that the results from ground corn starch[12] which disperses easily to give a limpid paste in hot water are practically the same as those from the thicker whole starch pastes.

As the ratio of "Superase" to starch is increased, a larger amount of fatty acids is liberated up to a certain point. Increasing the amount of "Superase" relative to the starch beyond that point causes no further splitting of fatty acid residues from the amylose.

The "Superase" produces reducing sugars from both corn alpha and corn beta amylose, so that its action on whole corn starch is not confined to the beta amylose alone. The average amount of reducing sugar from 500 mg. of whole starch is equivalent to 190 mg. of iodine and from the same weight beta amylose is equivalent to 202 mg. of iodine, but beta amylose constitutes only 85% of the whole corn starch.[2b] Therefore some of the reducing material must have come from the alpha amylose. During this breaking away of carbohydrate from the main portion of the alpha amylose under the influence of the "Superase" only a small portion of the combined fatty acids were freed.

Any residue, therefore, which is left unattacked will have a new ratio of carbohydrate to fatty acid which will be unlike the original. Beta amylose is completely hydrolyzed to reducing sugars so if whole corn starch is treated with "Superase" any unattacked residue must come from the α-amylose alone.

Acting on these observations a large amount of a 2% corn starch paste was treated with the requisite amount of "Superase" under optimum conditions for twenty-four hours and a residue obtained by centrifuging the mixture kept mold free with toluene. This residue after washing was dried, ground and extracted for ten hours in a Soxhlet apparatus with ether to remove any free fatty acids. Subsequently a sample of the oven-dry material was hydrolyzed completely with aqueous acid and the fatty acids liberated, extracted, weighed and their iodine number determined. This analysis of the carbohydrate–fatty acid compound showed that it contained a large amount of combined fatty acids, namely, 6.3%, whose iodine number was 60.7. The whole corn starch from which the alpha amylose had come showed 0.66% fatty acids (all in the alpha) whose iodine number was 95.8 while the alpha amylose separated from the same starch before the attack of the "Superase" contained 3.8% combined fatty acids.

Discussion of Results

In this work it has been shown that by the use of three methods, namely, hydrolysis by acid, by base and by amylase, the fatty acid groups com-

(12) Taylor and Beckmann, THIS JOURNAL, **51**, 294 (1929).

bined with the α-amylose portion of corn starch are preferentially liberated. In all three cases the unsaturated acid radicals are the more easily removed. This result may be accounted for in two ways. It may be due either to a general lesser stability of compounds of carbohydrates with the unsaturated acids (oleic and linolic) in comparison with those of palmitic acid, or to the fact that these unsaturated acid residues are linked at positions different from that of the saturated one on the carbohydrate molecule. Position isomerism is known in the case of glucose derivatives to be the cause of differences in ease of hydrolysis.

Summary

1. The fatty acids associated with corn starch are hydrolyzed preferentially by acid, by base and by lipase-free amylase.

2. The linking between the unsaturated portion of the fatty acids and the carbohydrate is less stable to hydrolytic agents than that between the saturated portion and the carbohydrate.

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The Action of Aqueous Alkali on Starches, Amyloses and Modified Starches

BY T. C. TAYLOR AND G. M. SALZMANN[1]

The most common form of chemical change which starches or amyloses undergo gives rise to reducing sugars and is therefore probably a scission of glucosidic linkings. During the early stages of these transformations rather large changes in the physical aspects of the starches, or more particularly the pastes made from these starches, are obvious without any great accompanying changes in reducing value as measured by any of the common methods. By allowing the hot aqueous alkali to act on the sample, however, and then neutralizing and determining the new reducing matter iodimetrically by a modified Willstätter method,[2] it was found possible to magnify small differences among the starches and amyloses that would not be significant when based on the initial reducing value alone. This was possible because it was found that when any starch or amylose had an appreciable initial reducing value the aqueous alkali produced many times that amount after treatment.

While some inquiry was made into the nature of these reducing sub-

(1) This is an abstract of a dissertation by G. M. Salzmann, presented to the Faculty of Pure Science of Columbia University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) (a) Willstätter and Schudel, *Ber.*, **51**, 78 (1918); (b) Goebel, *J. Biol. Chem.*, **72**, 802 (1927); (c) Kline and Acree, *U. S. Bur. Standards J. Research*, **5**, 1063 (1932).