[CONTRIBUTION FROM THE TAKAMINE LABORATORY.]

A METHOD OF TESTING THE AMYLOLYTIC ACTION OF THE DIASTASE OF ASPERGILLUS ORYZAE.

BY SELMAN A. WAKSMAN.

Received October 18, 1919.

In the action of diastatic enzymes upon starch, we differentiate between the amylolytic (amyloclastic, liquefying) power and the saccharogenic (saccharifying) power. We usually speak of diastase as one enzyme, but we might consider, more properly, this enzyme to consist of two classes of enzymes, one of which hydrolyzes the starch to soluble starch, dextrins and maltose (amylase) and the other hydrolyzes the maltose into dextrose (maltase), but, since both classes of enzymes are usually found in the same mixture and were not as yet separated from one another, they are looked upon as one group of enzymes or even one enzyme. But the very fact that the diastatic enzymes obtained from different sources do not reduce the starch alike to the same end products, as will be pointed out later, would lead us to doubt the validity of many comparative studies of different enzyme preparations based upon the determination of the same end product. Some diastases, notably the malt preparations, although acting readily on starch (strong amylolytic power) and hydrolizing it to dextrins and finally to maltose, do not hydrolyze the latter to any large extent into dextrose, while diastase obtained from *Aspergillus Oryzae* (taka-diastase), in acting upon starch, hydrolyzes it into dextrins, then to sugars and results in a mixture consisting of glucose and maltose, free from any dextrins.

A review of the various investigations on the action of diastase reveals the fact that, in most of these, the saccharogenic power of the enzymes, rather than the amylolytic, was measured, for various reasons. First of all, in determining the saccharogenic power, we are dealing with the end products of the action of the enzyme upon starch, namely the sugars, which are measured readily by various methods. While in the study of amylolytic power, we have to measure either the amount of starch that has been hydrolyzed and this is not very satisfactory; or to determine the disappearance of the starch reaction with iodine, which also is not entirely satisfactory, since we have to differentiate then between the starch paste, soluble starch, and even higher dextrins which result in the first step of the hydrolysis of starch. The other reason for measuring the sugar production by enzymes from starch is due to the fact that the action of diastase was directed towards producing sugars from starch both in the feeding experiments and the production of beverages, therefore it was natural to measure the saccharogenic rather than the amylolytic power.

The investigations presented here were conducted for the purpose of

finding the best methods suitable for the measurement of the action of diastatic enzymes used in the textile industry for the purification of the fabrics from starches and starch derivatives (process of desizing). Naturally, under these conditions, emphasis should be laid on the amylolytic power of the enzymes rather than on the saccharogenic power, since in the textile industry we are concerned with the first step of the action of the enzymes upon starch, namely, the dissolving of the starch or bringing it to such a state where it would dissolve in water rapidly and could be washed out, while the rapidity of sugar production is of little interest.[1]

Historical.

A complete review of the different methods used in the study of diastatic action upon starch is found in the series of papers published by Sherman and associates.[2] Briefly stated, the methods for measuring the saccharogenic action consist in measuring the amount of sugar produced by determining the reduction of Fehling's solution; these methods are collectively designated by Sherman and associates as "copper" methods. The amylolytic (amyloclastic) or liquefaction methods aim to measure the power of the enzymes to convert a definite amount of starch into products which give no longer the characteristic reaction with iodine and are designated by Sherman and associates as "iodine" methods. The only criticism to be submitted here on the so-called "saccharogenic" or "saccharification" methods is, as was pointed out above, the entire lack of differentiation between maltose and dextrose. This has been recognized by Davis and Daish,[3] who, utilizing the fact that "taka-diastase" converts starch only into maltose and dextrose free from dextrin, used this method for the determination of starch in plant tissues; both the maltose and dextrose were evaluated by measuring the reducing power of the starch digest and the rotating power. Knowing the rotatory and reducing powers of the two sugars and of the unknown starch digest, we can easily figure out the quantity of maltose and dextrose present, and, therefore, of starch digested.

The iodine methods consist in testing the disappearance of the blue color (indicating disappearance of starch) or of any color (disappearance of starch and certain dextrins), upon adding a drop or two of the mixture of a standard starch solution and enzyme to a dilute solution of iodine (see Johnson,[4] Wohlgemuth,[5] Sherman[6]).

[1] May, *J. Soc. Dyers and Colourists*, **27**, 88 (1912).

[2] THIS JOURNAL, **32**, 1073, 1087; **33**, 1195 (1911); **34**, 1104 (1912); **35**, 1617, 1784, 1790 (1913); **37**, 623, 643, 1305 (1915); **38**, 1638, 1877, 1885 (1916); **41**, 231 (1919).

[3] *J. Agr. Sci.*, **6**, 152 (1914).

[4] THIS JOURNAL, **30**, 798 (1908).

[5] *Biochem. Z.*, **1**, 9 (1908).

[6] THIS JOURNAL, **32**, 1073 (1910).

Experimental.

In this study only the liquefaction of the starch is taken up; the methods of Johnson, Sherman and associates, and others based upon the reduction of the starch by the enzyme to substances no longer giving any color with iodine were not suitable for the purpose which we here have in view, since these methods measure not the first step in the liquefaction of the starch but the total disappearance of the starches and dextrins; the difference between the saccharogenic methods and these iodine methods consist in the fact that in the first case the sugars are measured directly, while in the second the conversion of the starch into sugars is measured. The only other method, which actually measures the liquefaction of starch is that of Wohlgemuth and its various modifications, in which the disappearance of the blue color with iodine is taken as an end-point, but not a total absence of color with iodine. This method, based upon a degree of color, is subject to various criticisms, since it will vary in the hands of different investigators.

The method used here is based upon the disappearance of the starch (the so-called "raw starch" was used) in the solution, without considering the fact of just how much of the starch has been hydrolyzed to dextrins and how much into sugars, thus measuring as nearly as possible the first step in the hydrolysis of starch.

Since, as Sherman and associates[1] have recently shown, potato starch is obtained almost pure by washing with water only and is as readily acted upon in the water washed as in the more highly purified condition, this starch was used in the raw state. A 2% paste was prepared by mixing the proper amount of dry starch in a little cold water, then adding boiling distilled water, so as to form a uniform suspension, boiling it for 10 minutes and making up to volume with distilled water. The paste is introduced in 10 cc. portions into large test-tubes and these placed in a thermostat kept at 40°; as soon as the temperature is obtained, the proper amount of enzyme is added, in varying quantities, to the tubes, these are well shaken and placed back into the thermostat. As soon as the starch is hydrolyzed, the solution loses its opaque color and becomes clear; this is easily observed by comparing the liquefied and unliquefied tubes. This method can readily be subjected to criticism, since it will give slightly varying results in the hands of different workers, particularly beginners.

To make the end-point clearer, it was decided to add to the starch a coloring substance, which, not interfering with the action of the enzyme, will help recognize the disappearance of the colloidal starch paste. Out of a large number of coloring substances tested, *neutral red* was found to be most suitable. The material is prepared as follows: Place in a large porcelain dish 50 to 100 g. of dry potato starch, pour upon the starch

[1] THIS JOURNAL, 41, 1129 (1919).

100 cc. of 0.5% solution of neutral red, allow the starch to absorb all the color, then wash the colored starch repeatedly with water, till all the supernatant liquid becomes almost perfectly clear. The colored starch is then dried. A 2% paste of this starch is prepared by the method given before. When this colored starch is liquefied by the action of the enzyme, the change from the colloidal to the clear state is readily observed, if no turbid substances were introduced with the enzyme material.

Instead of taking one period of incubation, use is made of the law of the action of diastase upon starch; the product of concentration of the enzyme by the time of action is constant, at least within certain limits of enzyme concentration and time: $E \times T = K$. When the enzyme concentration is too low or too high or the time too short or too long, this law may not hold true. A unit of enzyme is taken as the amount of enzyme which will liquefy 10 cc. of 2% "raw" potato starch in 30 minutes at 40°. But, for practical purposes, it is more convenient to use such a concentration of enzyme that would liquefy the starch in not less than one minute and not more than 15 minutes; the tubes liquefied in periods of time outside of these limits should not be considered.

Example.—5 tubes[1] containing 10 cc. of the starch solution are placed in the thermostat and kept at 40°. The enzyme solution is diluted 10 times with distilled water (malt flour is extracted for 2 to 4 hours in 20 times the quantity of distilled water). Various quantities of the diluted enzyme are added to the tubes containing the starch solution. The tubes are shaken occasionally so as to insure a thorough mixing. As soon as the starch is liquefied the opaque color of the solution becomes clear (holding the tubes in the light). When the solution in a tube has cleared up, which can be more readily ascertained by comparing with the tubes containing the higher and lower quantities of the enzyme, the time is recorded. One test is given in Table I.

TABLE I.[a]

Concentration of enzyme in cc.....	0.1	0.2	0.4	0.6	1.0
Liquefaction, minutes.....	12½	6	3	2	Less than 1

Last tube is discarded.

$K = E \times T.$

E.		T.		K.
0.1	×	12.5	=	1.25
0.2	×	6	=	1.2
0.4	×	3	=	1.2
0.6	×	2	=	1.2
Av.,		K		1.21

[a] The observations reported in the table are individual determinations.

If $T = 30$ minutes, $E = \frac{K}{T} = \frac{1.21}{30} = 0.04$.

[1] The tubes used in this work were of heavy glass, 8″ long and 1″ in diameter.

Since the original solution of enzyme was diluted 10 times, we find that the enzyme value of one cc. $= \frac{1}{0.1 \times 0.04} = 250$ units

$$F \begin{matrix} 30 \text{ min.} \\ 40^\circ \end{matrix} = \frac{D \times t}{E \times T} = \frac{Dt}{K}.$$

F = enzyme value at 40° C. in 30 minutes; D = dilution; t = time of unit standard (t = 30 min.); E = quantity of diluted enzyme used; T = time of liquefaction. Thus by taking several tubes and averaging the products we can eliminate certain mistakes that will be made in the reading of the end-point. Given a denfiite amount of substratum and a definite temperature, the activity of the enzyme can be determined under different conditions of time.

When the stage of hydrolysis corresponding to this end-point, is tested with iodine, it is found to correspond to the point when no heavy blue color is obtained, but only a deep brown to faintly violet-brown color or faint blue-violet color showing that all the starch paste has been transformed into dextrins.

This method is particularly suitable for the study of enzymes that have a starch liquefying power rather large in comparison with the saccharifying power. Kendal and Sherman[1] have shown that, with pancreatic amylase, the hydrolysis of starch tended to come to an equilibrium or to become exceedingly slow when the weight of maltose reached about 85% of the initial weight of starch, even though the amount of enzyme was varied through a fairly wide range. Sherman and Baker[2] have shown that the ratio of the amyloclastic action to the saccharogenic is much higher for *Aspergillus Oryzae* diastase than for malt and pancreatic diastase. The iodine end-point occurred at a much earlier stage in the saccharogenic action, usually when from 20 to 30% of the theoretical yield of maltose had been produced. The *Aspergillus Oryzae* amylase exerts a more pronounced catalytic effect upon the earlier than upon later hydrolysis involved in the transformation of starch through dextrins into maltose. Malt amylase, on the other hand, apparently does not correspondingly catalyze the complete disruption of the material to which the iodine test is due, since the iodine end-point is found only at a much more advanced stage of sugar production with this than with either of the other amylases. The iodine end-point takes place in the case of malt diastase, only when the maltose production has reached from 65 to 95% of the theoretical yield of maltose.

Keeping in mind the fact that the diastase from *Aspergillus Oryzae* produces a good deal of glucose, while malt and pancreatic diastase produce

[1] THIS JOURNAL, **32**, 1087 (1910).

[2] *Ibid.*, **38**, 1885, 1638 (1916).

chiefly maltose and only traces of glucose (Sherman and Punett,[1] Davis and Daish[2]), which will tend to give a still higher "copper value" for the *Aspergillus* diastase, we would expect important differences between the amyloclastic and saccharogenic powers of this diastase on the one hand and that of malt and pancreatic diastase on the other hand.

The materials used in this study were malt flour (extracted with distilled water for 2 hours), liquid (sirupy) malt extract (Diastophor), and dilute extracts of enzyme from *Aspergillus Oryzae* (commercial name Polyzime). The Lintner values were obtained by the use of a modified Lintner method.

TABLE II.

Source of enzyme.	Lintner value.	$F_{40^\circ}^{30\ \text{min.}}$ Starch liquefying value.
Malt flour, No. 1	600	120
Malt flour, No. 2	600	175
Malt extract	450	100
Polyzime, No. 1	180	200
Polyzime, No. 2	178	275
Polyzime, No. 3	159	250
Polyzime, No. 4	170	200

The data brought out in Table II fully confirm the results of Sherman and his associates as to higher amyloclastic power in comparison with the saccharogenic power of the *Aspergillus Oryzae* diastase than the malt diastase. Comparing the results of the table which was made by one person on the same starch using the same methods, we find that, while the ratio of the liquefying value to the Lintner value of malt preparations was, under the conditions of the experiment, about $1/4$ to $1/5$, that of dilute *Aspergillus Oryzae* enzyme was 1 to 1.5, showing that the latter preparations have about 4 to 6 times as strong a liquefying power for starch in comparison with the saccharogenic power than the malt preparations.

This is in accordance with the investigations of Sherman and Tanberg,[3] who have shown that, while in the case of pancreatic amylase the ratio of the amyloclastic to the saccharogenic power is 2 : 1, in the case of *Aspergillus Oryzae* diastase, the ratio of amyloclastic to saccharogenic powers is 7 : 1 to 9 : 1.

Summary.

1. The Lintner method for measuring the saccharogenic action of different enzymes upon starch should not be used for comparative studies of different enzymes, since the end products are not the same in the case of the different enzymes.

2. The starch liquefying (iodine) methods with the exception of that

[1] THIS JOURNAL, 38, 1877 (1916).

[2] *Loc. cit.*

[3] THIS JOURNAL, 38, 1638 (1916).

of Wohlgemuth, do not give the "liquefaction" of starch by enzymes, but measure the reduction of the starch to substances giving no longer any reaction with iodine, which is arbitrary.

3. In the study of the action of enzymes upon the starches present or embodied in the textiles (process of desizing), the Lintner method and its various modifications are unsuitable, since we are interested not in the sugar production, but in the starch elimination.

4. A method is described for measuring the starch liquefying power (amyloclastic action) of enzymes, which is practical and easily manipulated.

5. Comparing the starch liquefying powers of malt enzymes and enzymes obtained from *Aspergillus Oryzae* (water extract used known commercially as Polyzime) the first are found to give a higher Lintner value, while the second give a higher liquefying value. The ratio of the liquefying value to the Lintner value, in the case of the malt preparations, is 1 : 4 to 1 : 5, while in the case of *Aspergillus Oryzae* enzyme it is 1 : 1 to 1.5 : 1, so that the liquefying power of the latter, in comparison to its sugar producing (saccharogenic power), is 4 to 6 times as great as that of the malt preparations.

CLIFTON, N. J.

[CONTRIBUTION FROM THE CHEMISTRY LABORATORY OF THE UNIVERSITY OF ILLINOIS.]

ORGANIC CHEMICAL REAGENTS. V.[1] THE PREPARATION OF ALKYL AND ALKYLENE BROMIDES.

BY OLIVER KAMM AND C. S. MARVEL.

Received November 13, 1919.

Although alkyl bromides are among the most common of laboratory reagents and although they are prepared by the most elementary reactions met with in organic synthesis their preparation in the laboratory as well as on a relatively large scale requires further improvement. The preparation of ethyl bromide is described in practically all laboratory manuals and yet the methods are so unsatisfactory that two papers[2] have appeared recently recommending improvements in the process, mainly in an endeavor to avoid the formation of ethyl ether as a by-product. In many reactions in organic chemistry, yields of from 70 to 80% are considered highly satisfactory but not so with the preparation of such simple and extensively used compounds as the alkyl halides. The method described below was found more adaptable to the preparation of alkyl and alkylene bromides than those methods commonly employed; it has been applied to the prep-

[1] For other papers in this series see THIS JOURNAL, 40, 1285, 1950 (1918); 41, 276, 789 (1919).

[2] *J. Chem. Soc.*, 107, 1489 (1915); 109, 1 (1916).