A Critical Investigation of the Amylolytic Activity of Pancreatin and Extract of Malt*

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The age of the product does not determine its amylolytic activity. A good appearing pancreatin eleven years old had a higher amylase activity than an off-colored, but recently manufactured, product. The official definitions of pancreatin and extract of malt are misleading. The official definitions should give the time involved in the assays in order to give a truer picture of their enzymatic activities.

KIRCHHOFF (in 1814) was the first to record the effect of a biochemical catalyst when he observed the influence of the glutinous component of wheat meal upon the conversion of starch to soluble carbohydrates. This phenomenon was later classified as enzymatic activity by Kühne (1878). A great deal of work has been done in determining amylolytic activity of substances since that time, yet the official method recorded in the United States Pharmacopœia for the amylase activity of Pancreatin and Extract of Malt is still subject to considerable criticism.

In order to evaluate the official assay method now in use a critical investigation of the substrate and its preparation, of the period of digestion, of the end point, of the temperature, and even of the official definition should be made. Starch, without indicating its source, is the conventional substrate used in most qualitative and quantitative assays. The Pharmacopœia specifies potato starch. Since the chemistry of the starches is not entirely understood, and since there are individual differences which manifest themselves in digestion experiments, the choice of a particular starch in the Pharmacopœia may be looked upon as worth while. The use of potato starch is based upon the findings of Graber (1). The theory that starches are made up of different components in varying proportions, some of which are attacked by one group of amylases and some by others, makes the choice of the substrate even more important. Without specifying any botanical variety, Van Klinkenberg (2) asserts that starch is a mixture of 36 per cent α-starch and 64 per cent β-starch. Kühn (3) does not subscribe to the "mixture theory." He believes that starch is a single molecule with a chain of alternating α and β glucosidic linkages. Here again, the nature of the amylase will determine whether it will attack the α or the β linkage which will materially influence the nature of the intermediary products.

It has been fairly well established that starch consists of two substances, α-amylose or amylopectin, found in the outer layer of the granules and not readily dispersible in water, and β-amylose found in the interior and dispersible in water. Various workers have separated these two components and have reported yields of soluble amylose varying from 17 per cent to 98 per cent depending upon the method of separation. According to Taylor and Iddles (4) the yield of α-amylose depends upon the botanical source of the starch. Potato starch gave the lowest yield of those varieties tried. This may explain why potato starch, according to Graber (1), is more readily converted into soluble carbohydrates by pancreatic amylase than are the other starches.

Kühn (3) made the important observation that pancreatic and salivary amylases act upon starch to give primarily α-maltose. He classified these enzymes as the *alpha* type. He named another group of amylases, obtained from sprouted and unsprouted grains, the *beta* amylases, since they act upon the same substrate to give a large yield of β-maltose.

Various methods are used for the estimation of amylolytic activity. Chief among these are:

1. Measurement of the reducing sugar

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formed as a result of the digestion of starch by an amylase.

2. Measurements of the decrease in viscosity of the reaction mixtures.

3. Observations of the changes in color given by the reaction mixtures when treated with iodine.

4. Determination of residual amyloses.

The United States Pharmacopœia recognizes the third method for the quantitative determination of amylase in Pancreatin and Extract of Malt. An important objection to this method of analysis is that the assay methods use different intervals of time for the digestion period. Although the interval of time is given in each of the respective assays, it is not indicated in the official definition of either. The quantitative values given in the official assays are indeed misleading.

The object of this investigation is to find a more satisfactory method of analysis for amylase activity than that given by the United States Pharmacopœia, and to suggest changes in the monographs for Pancreatin and Extract of Malt which will avoid the misinterpretations which are now commonly made. The present monographs lead many to believe that Pancreatin contains about five times as much amylase as Extract of Malt, but the true ratio is about 30 to 1.

EXPERIMENTAL

The potato starch paste made by the official method either under Pancreatin or Extract of Malt is a heterogeneous mixture which does not give uniform digestion. The color of the end point of the assay is very difficult to read accurately, and the error is thus magnified. Furthermore, no consideration is given to the influence of such factors as activators or the control of hydrogen ion concentration.

It was found that a more nearly homogeneous starch paste could be obtained if, after being boiled, it was run through a hand homogenizer several times. The homogenized paste is less viscous than the official paste. A small amount of starch is lost in the homogenizing, which can be corrected by adding 1% of potato starch to the original amount. If this is done the loss is negligible (about 0.1%).

An alternate method is to make a paste by boiling 5.625 Gm. of dry starch for five minutes with 125 cc. of distilled water in a tared beaker of 250 cc. capacity. It is cooled to room temperature and enough distilled water is added to make the mixture weigh 150 Gm. One hundred grams of this paste (containing 3.175 Gm. of dry starch) is used.

In the official assay of amylase activity on potato starch, the end of the reaction is indicated by the absence of a blue or violet color between the iodine and the starch digestion mixture. The starch digestion mixture reacts with iodine to give colors which vary from blue to violet, to various shades of red and pink; and as digestion proceeds they finally fade to the color of the standard iodine solution which is employed. The temperature of the iodine solution must be carefully controlled, since varying the temperature not only affects the time required to reach the official end point, but gives color variations peculiar to the temperature employed.

The assays were run according to the directions given in the United States Pharmacopœia with different temperatures of the iodine solution in order to determine the influence of this factor.

It was found that the temperature of the iodine test solution plays an important role in determining the end point. The tests were made at fifteen-second intervals.

The standard used was the blue solution obtained by adding 0.1 cc. of the digestion mixture (0.15 Gm. of pancreatin added to 3.75 Gm. of dry starch made into a paste and digested at 40° for five minutes) to the dilute iodine solution maintained at 24.8°.

At 12° the starch iodine solution showed a distinctly bluish green color which tended to go to blue. At 25° the solution was a decided blue, with a tendency toward purple as the digestion continued. At 38° the solution, even during the first few minutes of digestion, showed a red color which gradually faded.

Table I shows the digestion time that is necessary in order to get the color with iodine that matches the standard.

TABLE I.—SENSITIVITY OF THE STARCH IODINE REACTION TO TEMPERATURE CHANGE

No.	°C. of Reaction Mixture	°C. of Iodine Solution	Period, in Min., Digestion in Order to Reach the End Point
1	40	12.5	6.5
2	40	15.3	6.0
3	40	18.6	5.75
4	40	24.8	5.0
5	40	32.0	3.5
6	40	38.5	1.5

The United States Pharmacopœia states that the temperature of the iodine test solution should be "23° to 25°." It is apparent that this temperature is important and must be maintained for comparative work, for any variations in the temperature of the iodine solution will materially influence the results.

It was found to be very difficult to match the colors considered to be the true end point. However, the intensity of the colors could be compared satisfactorily.

The sensitivity of the homogenized starch iodine test was determined as follows: 1.0 Gm. of dry starch, accurately weighed, was made into a paste by boiling with 100 cc. of distilled water for five minutes. The paste was cooled to room temperature and homogenized, and distilled water was added to make 250 cc. Various dilutions of this preparation were made and treated with the dilute iodine solution. The tests were made at room temperature (23°). The dilute iodine solution was made according to the Pharmacopœia and consisted of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water.

While the starch iodine reaction could be detected in dilutions of 1 to 20,000 if observed instantly at the point of contact, it could not be detected when the two solutions were mixed as directed to be done in the Pharmacopœia. In fact, 1 part of starch in 2000 parts failed to give a positive test.

Thus a considerable quantity of starch may be undigested and still fail to manifest itself by the official method of identification.

Starch which had not been homogenized was also used. The difficulty of getting uniform dilutions and even distribution of the color produced made it impossible to record comparative values.

The Effect of Light on the Starch-Iodine Color.—Willson (5) suggested that a reference sample of U. S. P. Pancreatin be chosen as a standard, and that the assays of unknown pancreatin preparations be compared with it. It is important to note the influence of light upon any color standard of this character.

A typical blue color of the starch-iodine reaction was obtained by adding a small portion of a partially digested starch paste to the dilute iodine solution. This blue solution was divided into three portions.

Portion A was placed directly in the sunlight. Portion B was kept in the room away from the direct rays of the sun but in diffused light. Portion C was kept in a dark locker when it was not employed as the standard.

A comparison of portion C at the end of two hours with portion A at the end of the same period of time proved clearly that the color fades very greatly in direct sun light. This is true to a lesser extent in diffused light, yet it is significant in quantitative determinations, and indicates that the standard should not be made too far in advance.

Determination of a Satisfactory End Point.—Sherman, Kendall, and Clark (6) determined the end point of starch digestion by using larger amounts of digestion mixture and iodine in a smaller volume than in the official assay. This intensifies the color before the end point is reached and makes the end point, where there is "no color which can be distinguished from that of the untreated iodine solution," more definite.

Experiments were made to determine if this end point could be used in the official assay of Pancreatin with the amounts of digestion mixture and iodine solution called for by the Pharmacopœia.

The use of larger quantities of starch than directed in the Pharmacopœia was also tried to determine its value over a wide range of concentrations. Each succeeding assay contained 3.75 Gm. of additional dry starch diluted in the same ratio as before, with an increase in weight of 100 Gm. over that used in the preceding assay.

Digestion was considered complete, as directed in the Pharmacopœia, when "0.1 cc. of the digestion mixture, added to a previously made mixture of 0.2 cc.of 0.1 *N* iodine in 60 cc. of distilled water," showed as indicated by Sherman, Kendall, and Clark (6) "when viewed against a white background, no color which can be distinguished from that of the untreated iodine solution." This is the Sherman, Kendall, and Clark (6) end point using concentrations of digestion mixture and iodine solution called for by the Pharmacopœia.

TABLE II.—DETERMINATION OF THE TIME REQUIRED FOR THE DIGESTION OF VARYING AMOUNTS OF STARCH USING A MODIFIED SHERMAN, KENDALL, AND CLARK (6) END POINT

No.	Mg. Pancreatin	Gm. Dry Starch	Total Wt. of Mixture, Gm.	Time, in Min., Required for Digestion
1	150	3.75	100	8
2	150	7.50	200	20
3	150	11.25	300	36
4	150	15.00	400	50
5	150	18.75	500	61
6	150	22.50	600	75
7	150	26.25	700	101
8	150	30.00	800	109

It will be recalled that in the official method the end point is considered reached when no blue or violet color is produced, but the allowance of some color makes it very indefinite.

It takes the digestion mixture a longer period of time to attain the end point of Sherman, Kendall, and Clark (6) than that given in the Pharmacopœia for its end point. However, it was found that the eight minutes required to reach the end point by this method is comparable to the amount of Pancreatin which takes five minutes by the method of the United States Pharmacopœia, and is much easier to see.

The experimental data giving the time required to reach this end point not only by using the amount of starch indicated in the Pharmacopœia but in greater concentrations are recorded in Table II, from which it appears that reaction number 1 is the best to use.

A Modification of the Official Assay.—Upon the strength of the experimental evidence concerning the use of homogenized starch, and the modification of the Sherman, Kendall, and Clark (6) end point, the following changes were made in the official assay:

Instead of 3.75 Gm. directed in the official assay, 3.788 Gm. of homogenized potato starch was used.

The total weight of the mixture was increased from 100 to 150 Gm. to allow for the rinsing of the homogenizer.

Water, previously heated to boiling, instead of the temperature of 50° to 60° was found to hasten the gelatinization of the starch and to prevent the formation of a tough paste at the bottom of the beaker. This paste is not only difficult to homogenize, but is not readily digestible.

The modified end point of Sherman, Kendall, and Clark (6) was employed. The italicized portion of the following monograph indicates the extent to which the official assay was modified.

"Determine the percentage of moisture in potato starch by drying 0.5 Gm. accurately weighed at 120° for four hours. Thoroughly mix a quantity of the starch, equivalent to *3.788 Gm. of dry starch* (*3.75 Gm. plus* 1%) with 10 cc. of cold distilled water. Add the mixture with constant stirring to 75 cc. of distilled water, *previously heated to boiling*, contained in a 250 cc. beaker. Rinse the remaining starch into the beaker with 10 cc. of distilled water, and boil it gently for five minutes or until a translucent uniform paste is formed. Cool to room temperature. *Run it through a hand homogenizer three times, collecting it in a tared 250 cc. beaker. Pour several 20 cc. portions of hot distilled water into the homogenizer, in order to remove the remainder of the starch paste, and add the rinsings to the paste in the beaker. Add enough distilled water to make the mixture weigh 150 Gm.*

"Warm the paste to 40° and place the beaker in a water bath maintained at 40°. Suspend 0.15 Gm. of pancreatin in 5 cc. of distilled water and add the suspension to the starch paste, mixing it well by pouring the mixture from beaker to beaker for 30 seconds, noting the time when the pancreatin suspension was added to the starch. Maintain the mixture at 40° for exactly *eight* minutes. At once add 0.1 cc. of the starch pancreatin mixture into a previously made mixture of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water. *When viewed against a white background, there should be no color which can be distinguished from that of the untreated iodine solution.*"

The Preparation of β-Amylose.—Sherman and Baker (7) found that pancreatic amylase produced sugar more rapidly from β-amylose than from α-amylose. They also found that in the early stages of digestion malt amylase showed a greater yield from α-amylose than from β-amylose.

In order to see if any appreciable difference in the activity of pancreatic amylase could be obtained by using β-amylose in place of whole starch, a method of obtaining this component was needed. The literature gives various methods which are employed for the separation of the components of potato starch.

Gruzewska (8) effected a separation by treating potato starch with dilute alkali, and after neutralization and sedimentation, decanting the dissolved amylose from the residue of amylopectin. The amylose represented 40–45% of the original starch.

By treatment of potato starch with hot water, sedimentation, and decantation, Tanret (9) estimated 27% of amylose.

Sherman and Baker (7) subjected a thin paste of potato starch to centrifugal force, and obtained about 15% in *beta* amylose form.

Samec and Mayer (10) *electrodialyzed* a dispersion of potato starch prepared at 120°. The amylose fraction represented 17%.

Ling and Nanji (11) *fractionated* potato starch by freezing a paste, and after melting the frozen mass, separated the solution of amylose from the amylopectin by centrifuging.

Taylor and Iddles (4) disintegrated starch grains, previously treated with hydrogen chloride in alcohol, with ammonium thiocyanate solution, then precipitating with alcohol and subjecting the precipitate to ultra-filtration or dialysis. The yield of soluble amylose was 97–98%.

Baldwin (12) effected separation by gelatinizing, freezing, and performing a series of extractions.

A rapid and satisfactory method was found which consisted of boiling a 2.5% starch paste for thirty minutes to rupture the granules. The paste was then precipitated with alcohol, dried, and powdered. The soluble amylose was separated from the insoluble component by allowing to stand in cold water, decanting, and boiling the aqueous solution to concentrate the β-amylose. The insoluble α-amylose was washed free of β-amylose, precipitated, dried, and powdered. A yield of about 36.5% of β-amylose was obtained.

A Detailed Method of Separating Potato Starch into Alpha and Beta Amylose.—Thoroughly mix a quantity of starch equivalent to 10.0 Gm. of dry starch U. S. P. with 50 cc. of cold distilled water, and add, with constant stirring, to a sufficient amount of boiling distilled water to make 350 cc. Rinse the remaining starch into the beaker with two 25-cc. portions of distilled water and boil gently with constant stirring for thirty minutes. Add distilled water when necessary to maintain the original volume. Allow the paste to cool to room temperature. Transfer the paste to a 2000-cc. Erlenmeyer flask, using 50 cc. of distilled water to remove the remainder of the paste from the beaker. Now superimpose over the starch paste 500 cc. of 95% alcohol, stopper the flask and shake vigorously until the starch is completely precipitated. Filter and allow the precipitate to dry for twelve hours at 65°. Grind the dry altered starch in a mortar, and pass the powder through a number 100 sieve.

Weigh about 5 Gm. of the altered starch and sprinkle it with constant stirring into 500 cc. of cold distilled water in a 2000-cc. beaker. Add enough water to this mixture to make the final volume measure about 1000 cc. and let it settle for twenty-four hours. Decant the clear supernatant liquid and concentrate it by boiling to a volume of about 200 cc. or until the β-amylose will be precipitated with an equal volume of alcohol. Precipitate the β-amylose with 200 cc. of 95% alcohol and dry it at

65°. Powder the precipitate so that it will pass through a number 100 sieve. This was used as the β-amylose in succeeding work.

Saccharogenic Activity of Pancreatin Using Different Substrates.—In addition to β-amylose, potato starch, corn starch, and soluble starch were used.

The method of analysis used was based on the amount of maltose produced from a given amount of substrate in a given unit of time. It is essentially the method of Willstätter, Waldschmidt-Leitz, and Hesse (13). These authors used soluble starch as the substrate. The different substrates being tested were prepared by making one per cent pastes and boiling for five minutes.

It was found that the different substrates gave no appreciable difference in the amount of maltose produced. There appears to be no advantage in using β-amylose for this determination. If any conclusions could be drawn from these findings, the chief one would be that β-amylose is slightly inferior to the other substrates used. The experimental data are recorded in Table III.

TABLE III.—SACCHAROGENIC ACTIVITY OF PANCREATIN USING VARIOUS STARCH SUBSTRATES

Substrate	Mg. Pancreatin	Cc. 0.1 *N* Iodine	Mg. Maltose
Homogenized potato starch	5	4.09	70.14
Potato Starch	5	4.15	71.17
Corn Starch	5	4.27	73.23
Soluble Starch	5	4.07	69.80
β-Amylose	5	3.40	58.31

The Amylase Activity of Different Samples of Pancreatin.—During the course of the experiments it was noted that many of the older samples of pancreatin had caked and had assumed a darker color than usual. Experiments were made to compare the amylolytic activity of the older samples with several fairly fresh samples of pancreatin.

In order to determine accurately the loss in amylolytic activity due to aging, it would be necessary to store a sample of pancreatin under a certain set of conditions, and to determine its activity from time to time. Since this was impractical, several commercial samples of pancreatin were obtained from different drugstores in the vicinity. These samples varied in age from eleven months to fifteen years and came from three different pharmaceutical houses. The conditions under which the samples were stored no doubt varied considerably, since some of the samples were typical cream-colored powders, while other samples were caked, and of a deep brown color.

The activities of these samples of pancreatin were tested by the method of Willstätter, Waldschmidt-Leitz, and Hesse (13). The only change from the original assay was that a 1% homogenized potato starch paste, which had been boiled for five minutes, was used in place of the soluble starch suggested by the authors.

One hundred milligrams of pancreatin was suspended in 100 cc. of distilled water, and 5 cc. of this suspension was added to the substrate.

The pancreatin assayed by the above method showed an irregular drop in amylase activity as the ages of the samples increased. These findings fail to establish any reasons for the irregularity in amylase content. It appears that the age of the sample is of minor consequence, since properly stored samples, judged by their physical appearance, of 16, 53, 60 106, and 132 months of age had practically the same enzyme activity. The samples which were caked and brown in color gave a lower amylolytic activity. Whether these samples were inferior to begin with or changed in appearance and amylolytic activity because of improper storage could not be determined. Nevertheless, it appears that Pancreatin which has caked or is dark brown in color does not have the amylolytic activity which is found in the dry, powdery, light-cream-colored samples.

The experimental work is tabulated to show the source of pancreatin, as derived from companies "A," "B," and "C," and may be seen in Table IV.

TABLE IV.—DETERMINATION OF THE AMYLASE ACTIVITY OF SAMPLES OF PANCREATIN FROM DIFFERENT COMPANIES

Co.	No.	Age Mo.	Cc. 0.1 *N* Iodine	Mg. Maltose
A	1	39	4.53	77.69
A	2	53	4.68	80.26
A	3	92	4.58	78.55
A	4	98	5.58	78.55
B	5	60	4.47	81.29
B	6	60	4.74	81.29
B	7	96	4.69	80.43
B	8	120	3.14	53.85
B	9	132	4.64	79.58
B	10	156	4.02	68.94
C	11	15	3.55	60.88
C	12	16	4.74	81.29
C	13	28	3.24	55.57
C	14	47	3.19	54.71
C	15	69	4.12	70.66
C	16	82	4.58	80.55
C	17	133	0.67	11.49
C	18	142	0.31	5.32
C	19	180	0.31	5.32

SUMMARY

1. The heterogeneous character of the potato starch paste in the United States Pharmacopœia for the assay of amylase activity under Pancreatin makes it very difficult to get uniform reaction mixtures. This condition may be greatly improved by homogenizing the starch paste.

2. The starch iodine end point of the official assay is unsatisfactory for the following reasons:

(*a*) One part of starch in a 2000 dilu-

tion cannot be differentiated from the standard iodine solution.

(*b*) The starch iodine reaction is very sensitive to light.

(*c*) The temperature of the iodine solution not only influences the color and intensity, but also the time involved in reaching the end point.

(*d*) The end point is not a true indicator of amylase activity, since it does not register the termination of this hydrolytic reaction.

3. Pancreatic amylase is relatively stable over long periods of time when properly stored. Samples of good appearance varying in age from sixteen months to eleven years had practically the same enzyme activity. Other samples of similar age but of poor appearance had a much lower activity.

4. If the official assay is retained, it is recommended that the following modifications of the official monograph of Pancreatin be made:

(*a*) The starch paste used should be homogenized. There is no good reason to insist on the use of potato starch.

(*b*) The wording of the end point "*no blue or violet color is produced*" be changed to *no color is produced which can be distinguished from that of the untreated iodine solution.*

(*c*) The official definition of amylase activity, wherein it states "*Pancreatin converts not less than 25 times its weight of starch into soluble carbohydrates,*" be modified by adding the phrase, "*in 5 minutes.*"

(*d*) It is further suggested that these modifications be observed in the official assay of Extract of Malt and that the definition of amylase activity for this preparation be modified to read, *Extract of Malt is capable of converting not less than five times its weight of starch into water soluble sugars in 30 minutes.*

5. The modifications suggested in 4 *c.* and *d.* should be adopted in order to avoid the misinterpretation which is commonly made because the time element is not conspicuously recorded.

6. The substitution of different starches or components of starch for the soluble starch by the method of Willstätter, Waldschmidt-Leitz, and Hesse (13) gave practically the same pancreatic amylolytic activity. This is an advantage over the official method which specifies the use of *potato starch.* Furthermore the use of activators, the control of *p*H, and the determination of the amount of maltose as the end product give this method added advantages over the official method.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

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Phosphopyruvic acid
dl-Phenylglyceric acid
Pregnanediol
Isoandrosterone
Dehydroandrosterone
2-Aminobutyrolactone hydrobromide
5-Chloropentanone-2
Emetine
Dimethylhydroxylamine
d- and *dl*-Cysteine hydrochloride