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Investigations on Malt Amylase

III. On the Colorimetric Determination of α-Amylase

ESKIL HULTIN

Biokemiska Institutet, Stockholms Högskola, Stockholm, Sweden

Several investigations have been performed on the colour reaction between starch and iodine, and several methods have been proposed making use of this reaction for the determination of amylase activity. This paper is intended to discuss some aspects of this subject and to give an account of a rapid method for the colorimetric determination of α-amylase.

A quantitative colorimetric assay of amylase was first described by Roberts [1], who measured the time necessary for the break down of a starch solution — prepared in a certain way — to the achroic stage and calculated amylase activities therefrom.

Hanes and Cattle [2] investigated the alteration in iodine coloration during the action of various amylases on starch. The extinction was measured at wave-lengths between 4300 and 7500 Å. The wave-length for maximum absorption was found to change towards shorter wave-lengths as the break down proceeds from about 6000 Å for soluble starch. Hanes and Cattle found a filter corresponding to the wave-length 5700 Å to be suitable for the colorimetric assay of amylase. This is due to the fact that they followed mainly the early part of the break down.

By plotting extinction values at 5700 Å against the corresponding values for the reducing power of the starch solution submitted to hydrolysis by various amylases, these authors found pronounced differences between the starchsplitting enzymes and even between α-amylases of varying origin. They explained this by the different capacities of the various enzymes to attack the large starch molecules and the smaller dextrin molecules.

Bernfeld and Fuld [3] have recently compaired various α-amylases by calculating the quotient saccharogenic activity divided by colorimetrically deter-

minated dextrinogenic activity, but did not find any differences in the early part of the break down.

A difference in the capacity of the various α-amylases to break down large and small polysaccharide molecules has been demonstrated quantitatively by Myrbäck and co-workers [4–7]. Their results seem to indicate that differences in the capacities of the reactiou products to give coloured compounds with iodine may be expected first at a late stage in the depolymerization.

It has been reported in several papers, *e. g.* those by Blom, Bak and Braae [8] and especially by Hanes and Cattle [2], that in the colorimetric determination of malt amylase the enzymic action depends on both the α- and the β-amylase.

The influence of various quantities of β-amylase on the results of colorimetric assays of malt α-amylase was investigated by Sandstedt, Kneen and Blish [9] *. They found that on the addition of increased amounts of β-amylase, the time necessary for the break down to a certain stage first diminished rapidly, but soon reached a value where addition of more β-amylase had no appreciable influence.

Thus, by keeping the amount of β-amylase sufficiently high in the reaction mixture, it is possible to get an assay of the amount of α-amylase in a mixture of α-and β-amylase.

Moreover, it might be possible (but probably not practical) to assay the β-amylase colorimetrically in solutions free from α-amylase by preparing a reaction mixture from a given amount of α-amylase, the β-amylase solution and starch solution.

In the last few years several authors [12–19] have proposed various methods for determining a suitable stage in the break down up to which the reaction time is counted. In order to render colorimetric amylase determinations comparable, a conversion factor should always be included, giving the capability of the enzyme to form reducing sugars, *e. g.* milliequivalents reducing sugars per minute and gram, or maltose equivalents. It must be observed [15, 20] that the time necessary for the break down of solutions of starch of different initial degree of polymerization differs somewhat.

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* They characterize their method as a standardized Wohlgemuth procedure. This seems to the present author to be erroneous. Wohlgemuth's method [10] is characterized by the simultaneous running of several experiments with various amounts of amylase for a constant time. Roberts [1] on the other hand, withdraws samples at suitable times from one reaction mixture. Roberts' method is furthermore the earlier. It is evident from this that the standardized method should be regarded as a modification of Roberts' method. This is true also of Ehrnst and coworkers' procedure [11, 12].

EXPERIMENTAL

On the shape of the extinction curves

The works of the present author on the colorimetric determination of α-amylase aimed first at finding out how a high degree of accuracy could be attained in a simple way. It is thereby desirable to get the same relative accuracy irrespective of the amount of α-amylase. This can be effected in the way suggested by Wohlgemuth [10] by preparing several reaction mixtures with the amylase activities forming a geometric series [10, 21]. Another way is to take the samples at times, forming a geometric series. A time table was thus prepared, in which the logarithm of the quotient of one time divided by the preceding was always 0.1. A table for more accurate determinations was also prepared, the logarithm of the quotients being 1/30.

The selection of a suitable colour standard for the determination of the end point is rendered difficult by the fact that the wave-length of the adsorbtion maximum is displaced during the break down towards or perhaps even outside the violet part of the visible spectrum [2]. In order to avoid this difficulty and to treat the problem more accurately, the extinction coefficient of the solutions were determined with a step-photometer, Leitz' Leifometer. The time necessary for the break down until the extinction at 5300 Å was 1, *i. e.* the transmission of light through a 1 cm layer was 10 %, was chosen as the reaction time.

The reaction mixture was made up of 3 ml of enzyme solution, 5 ml of an acetate buffer solution, an appropriate amount of a stock starch solution to render the reaction mixture 1 %, and water to 50 ml. In certain experiments part of the water was exchanged for appropriate amounts of β-amylase prepared as in Ohlsson [21].

The stock starch solution was prepared from Lintner [22] starch suspended in cold water and poured into boiling water. It was preserved in a flat-bottomed flask fitted with a stopper with an adapter and a glass tube leading from the bottom of the flask to the side tube of a burette. The system was sterilized by steam and the adapter and the burette plugged with cotton. Thus the starch solution could easily be preserved for some time and an appropriate amount easily withdrawn. The starch concentration of the solution — about 2 % — was determined from the dry weight of an aliquot part of the solution. When solutions were prepared from this stock solution, the solution withdrawn was first heated until it became quite clear.

The iodine reagent was prepared as suggested by Sandstedt, Kneen and Blish [9], and 1 ml of the reaction mixture added to 5 ml of the iodine solution. The extinction was read at about 20° C.

In three series, the apparent activities of α-amylase solutions were determined in the presence of various amounts of β-amylase. The results are given in Table 1.

Table 1. Determination of α-amylase activity in the presence of various amounts of β-amylase.

α-Amylase solution %	β-Amylase activity of the reaction mixture mg maltos/min · ml	Apparent α-Amylase activity mg starch/min · ml
0.08	0	11
	0.2	14
	1.0	30
	2.4	41
	4.9	48
	9.7	62
0.008	0	0.09
	0.2	4.2
	2.4	5.8
	4.9	6.2
0.0008	0	0.06
	0.2	0.65
	2.4	0.66

The concentration of the α-amylase solution is given in % of dry preparation, manufactured as described earlier [23]. The β-amylase activity of the reaction mixture was calculated from the activity and the amount of the β-amylase solution added.

By plotting the extinction coefficients E against the logarithms of time in these experiments, Figure 1 was obtained. In order to faciliate a close comparison of the three series with different amounts of α-amylase, the time notations in the series with the 0.008 % solution was divided by 10 and in the series with the 0.0008 % solution by 100.

When the extinction coefficients are plotted against log time and not against time, curves from assays of amylase solutions of different activities can be compared. In Fig. 1 the extinction coefficient curves from experiments with various amounts of α-amylase and β-amylase have the same shape if they correspond to apparent activity values that are the same fraction of the highest possible activity (in the presence of enough β-amylase).

From Fig. 1 we also see that to achieve considerable accuracy in the determinations it is desirable to take samples and colorimeter readings from the reaction mixture at times forming a geometric series in which the logarithm of the quotient is 1/10 to 1/30.

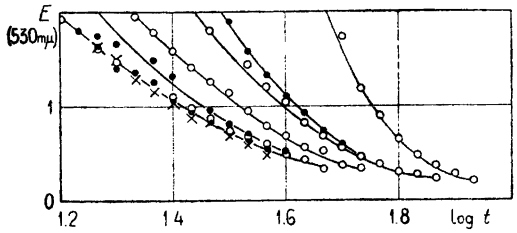

Figure 1. The extinction at 530 mμ as a function of log time with various amounts of α- and β-amylase.
The series of rings correspond to the activities 62, 48, 41 and 30. The series of dots correspond to the activities 6.2, 5.8, and 4.2. The series of crosses corresponds to the activity 0.66.

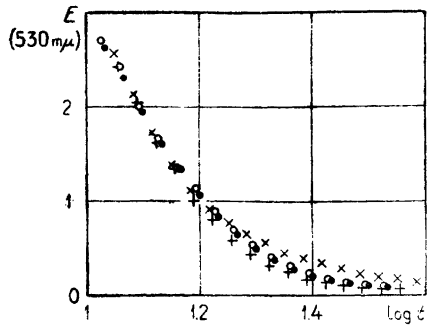

Figure 2. Extinction against log time curves from the break down of starch with malt α-amylase (dots), pancreatic amylase (rings), mould amylase (crosses ·) and salivary amylase (crosses –).

Comparison of extinction curves using various α-amylases

Some experiments were performed in the manner described with malt α-amylase, pancreatic amylase, mould amylase (Takadiastase) and salivary amylase in order to compare the shape of the extinction curves in the final part of the break down. The extinction values were plotted against the time, and to faciliate a close comparison, a suitable constant was added to the logarithm of the time notations in order to render the initial parts of the extinction curves coincident. The results given in Fig. 2 indicate that the differences in the action on starch by the various amylases give raise to slight differences in the shape of the extinction curves in the part near the achroic stage.

The influence of the initial molecular weight of the starch

The rapid method described later in this paper was used in these experiments. Two starch solutions were prepared, from common potato starch and from a lot of potato starch treated with 1.5 *N* hydrochloric acid, washed and dried. The molecular weight of the two lots were determined from viscosi-

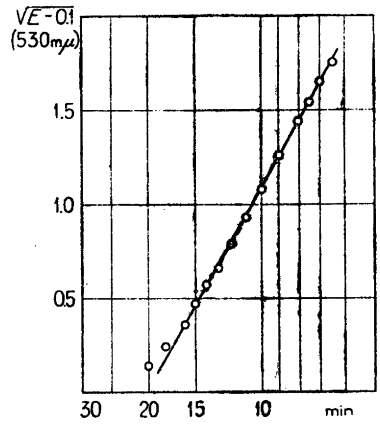

Figure 3. $\sqrt{E-0.1}$ *plotted against the logarithm of the time. It should be observed that the inclination of the line is always the same.*

metric measurements after extrapolation to zero concentration, using Staudinger and Husemann's value for the viscosity-molecular weight constant $K_m = 0.63 \cdot 10^{-4}$.

The mol. wt. and the time necessary for the break down until the extinction $E = 1$ was reached are given in Table 3.

Table 3. Comparison of the times required for the enzymatic breakdown of starch of various molecular weights.

M	t min
470 000	25.3
27 000	21.3

It is evident that the reaction time is influenced both by the initial and the final polymerization degree of the starch, and that it is rather vague to speak about the amount of starch broken down in colorimetric amylase assays.

A rapid method

For a rapid assay of the activity of amylase solutions containing no β-amylase, the present author has developed the following procedure, which renders the simultaneous performance of 5 assays possible.

2 ml of enzyme solution were pipetted into each of five 100 ml Erlenmeyer flasks and placed in a thermostat at 30° C. A discussion on the necessary accuracy of the temperature assay is given in a previous paper [24] by the present author. A 1 % buffered starch solution, made up from a suitable amount of a stock starch solution, buffer and water, was also preserved in the termostat. A stop watch was started, and at the time 1 minute 20 ml of the buffered starch solution was pipetted into the first Erlenmeyer flask and the flask shaken. When the stop watch showed 2 minutes, 20 ml was pipetted into the second flask and so on. 1 ml of the reaction mixture was withdrawn from each flask 10, 15, 25, 40 and 60 minutes after the starch had been added (if necessary) and poured into 5 ml of the iodine solution. The extinction coefficient was measured at 5300 Å for the solutions suitably coloured.

The moment corresponding to the extinction coefficient $E = 1$ could easily be obtained with tolerable accuracy in the following way. The square root — calculated on a slide rule — of the extinction coefficient was plotted on logarithm paper against the time (*i. e.* the square root plotted against the logarithm of the time). Then an almost straight line is obtained, and the time corresponding to the square root = 1 was read off. A still better result was obtained if the square root of the extinction coefficient minus 0.1 was taken, and the time read off as corresponding to the value 0.95 of this root. The values obtained from a control series are shown in Fig. 3.

Hanes and Cattle [2] found that the extinction decreases linearly with prolonged breakdown at the early part. That the square root of the extinction decreases linearly during some later part of the breakdown is only due to the fact that the reaction is retarded towards the end. If this happens at a stage where the colour may easily be assayed, one can make use of it to faciliate the calculation.

A conversion factor was determined by running assays of a malt α-amylase solution colorimetrically according to the method given here and by iodimetric determinations of reducing sugars according to Blom and Rosted's [25] modification of Linderstrøm-Lang and Holter's [26] method. The amylase amount 1 mg starch/min corresponded to 0.14 mg maltose/min if no β-amylase was present.

The principles of this procedure may of course also be applied to the Sandstedt, Kneen and Blish [9] method. The buffered β-amylase limit dextrin solution is then substituted for the buffered starch solution.

SUMMARY

1. A short review is given of the colorimetric assay of amylase. Some methods said to be modifications of Wohlgemuth's method should instead be characterized as modifications of Roberts' method.

2. If the extinction values are plotted against the time in colorimetric assays with various amounts of α- and β-amylase, only curves corresponding to activity values that are the same fraction of the highest possible activity (in the presence of enough β-amylase) have the same shape.

3. Extinction/log time — curves for α-amylases of different origin differ somewhat at the latest stage of the break down.

4. A rapid procedure for the simultaneous running of 5 colorimetric assays is described. 20 ml of a 1 % buffered starch solution is added at intervals of 1 minute to Erlenmeyer flasks containing 2 ml of enzyme solution. Samples are withdrawn after 10, 15, 25, 40, and 60 minutes if necessary and pipetted into iodine solution. Extinction values E are taken at 5300 Å. The values of $\sqrt{E-0.1}$ are plotted against the logarithm of the time and lie on a straight line at a suitably late stage of the break down. If the reaction time is counted until $E = 1$ (corresponding to $\sqrt{E-0.1} = 0.95$), the amylase amount 1 mg starch/min corresponds to 0.14 mg maltose/min.

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