ACTA CHEMICA SCANDINAVICA 3 (1949) 697–707

Investigations on Malt Amylase

II. On the Viscosimetrical Determination of α-Amylase

ESKIL HULTIN

Biokemiska Institutet, Stockholms Högskola, Stockholm

In a previous paper[1] the present author deduced the following formula for the enzymatic depolymerization of polymeric homologous substrates, if all linkages between the primary molecules are broken with equal ease and if the number of linkages broken is small in comparison with the number of linkages at complete polymerization:

$$A^{t^\circ}_{e|s} = c_s^2 \cdot \frac{\mathrm{d}\,\frac{1}{\eta_{sp}}}{\mathrm{d}t} \qquad (1)$$

where

$A^{t^\circ}_{e|s}$ = the enzymic activity at t° C (units per gram) in the reaction mixture; e and s are abbreviations for the names of the enzyme and the substrate (the indices may be omitted when there is no risk of confusion),
η_{sp} = the specific viscosity,
c_s = the concentration of the substrate in grams per gram reaction mixture, and
t = the time in seconds.

This formula was based upon Staudinger's equation

$$\eta_{sp} = K_m c_{gm} M \qquad (2)$$

where

K_m = the viscosity-molecular weight constant,
c_{gm} = the concentration of the substrate in basic moles per litre, and
M = the molecular weight.

For substrates, for which this linear relationship is not valid, but for which the modified Arrhenius-Staudinger formula (η_r = the relative viscosity)

$$\ln \eta_r = K_m c_{gm} M \tag{3}$$

can be applied, another formula has recently been deduced by the present author [2]:

$$A^{t^\circ}_{e/s} = c_s^2 \cdot \frac{\mathrm{d}\,\dfrac{1}{\ln \eta_r}}{\mathrm{d}t} \tag{4}$$

Equations (1) and (4) also define a unit A for the enzyme assay. In most cases, however, the unit $\mu A = A \cdot 10^{-6}$ is more convenient [3].

CALCULATION OF THE ACTIVITY

In addition to the guidance given in the first report [4] of this investigation, the following procedure is recommended.

For the calculation of the activity of an enzyme solution we employ the following notation:

m_e = grams enzyme solution mixed with the substrate solution,
m_s = grams substrate solution mixed with the enzyme solution,
C = the concentration of the stock substrate solution (grams substrate per gram solution).

The activity of the enzyme solution in the units $\mu A/g$ is then

$$\frac{m_s^2 C^2 \cdot 10^6}{m_e(m_e + m_s)} \cdot \frac{\mathrm{d}\,\dfrac{1}{\eta_{sp}}}{\mathrm{d}t} \tag{5}$$

and

$$\frac{m_s^2 C^2 \cdot 10^6}{m_e(m_e + m_s)} \cdot \frac{\mathrm{d}\,\dfrac{1}{\ln \eta_r}}{\mathrm{d}t} \tag{6}$$

respectively.

In calculating the activity much time can be saved if one always uses substrate solutions of the same concentration and calculates once and for all the factor $m_s^2 C^2 \cdot 10^6 / m_e(m_e + m_s)$. If one aims at the highest possible accuracy, one should calculate a table of this factor as a function of m_s and C. The

amount m_e of enzyme solution may be assumed to be measured with fairly good accuracy, but the amount m_s of substrate solution depends rather on the viscosity of the substrate and the drainage time of the pipette. The concentration of the stock starch solution may differ a little from the intended value. As an example a short table of the factor is given here as Table 1.

Table 1. Short table of the factor for calculation of enzymic activity. $m_e = 3.00$.

$100\ C$ / m_s	2.496	2.498	2.500	2.502	2.504
29.91	5645	5654	5663	5672	5681
29.94	5651	5660	5669	5679	5688
29.97	5657	5667	5676	5685	5694
30.00	5664	5673	5682	5691	5700
30.03	5670	5679	5688	5697	5706

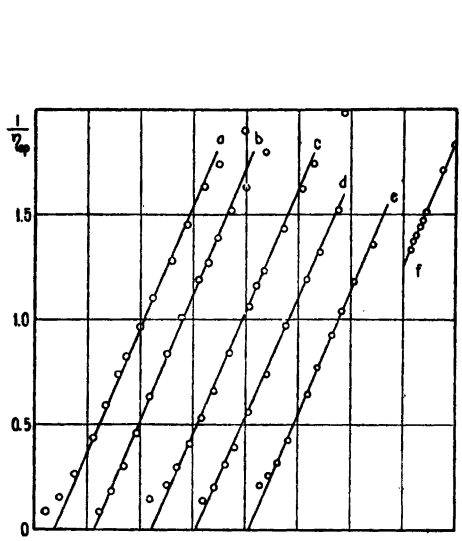
The calculation of the specific viscosities was discussed in the first report of this investigation. Tables 1 and 2 there are calculated for different concentrations of the substrate solution. Otherwise they give the same number $\tau_0 = \eta_w/dk_\tau$ *. For Table 1 only one decimal is required, but for Table 2 two decimals are often necessary. Sometimes sufficient accuracy is attained if the table is calculated so that the second decimal is an even number or even if it assumes the values 0 and 5. The relative viscosities are obtained similarly to τ/τ_0.

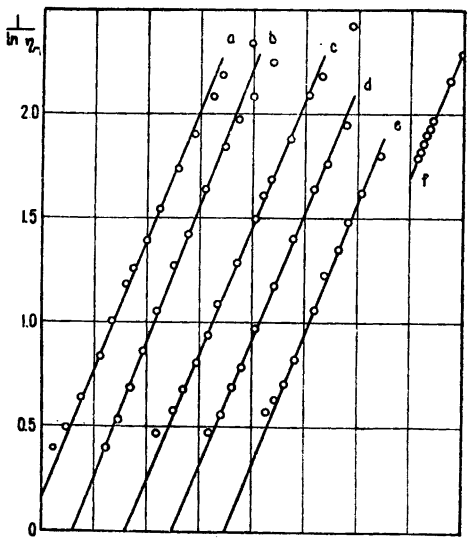
THE VISCOSITY OF THE SOLVENT

In calculations of the specific viscosities of starch solutions at different times, the solvent was referred to as water. However, distilled water is not actually used for viscosity measurements but buffer solutions; in the present investigation a buffer solution of sodium acetate and acetic acid, being 0.16 N in regard to sodium acetate and 0.04 N in regard to acetic acid. Hence, the viscosity of the buffer solution should be used in the calculations instead of the viscosity of distilled water.

The relative viscosities of sodium acetate and acetic acid solutions at 25° C were measured by Reyher [5]. On assuming the changes in the relative viscosities with the temperature to be negligible in these experiments, the relative viscosity of the present solvent in the stock solution may be calculated from

* d is here substituted for D in that paper.





Figs. 1 and 2. Graphs for amylase activity assay (see Table 2).
The distance between the lines cutting the time axis corresponds to 1000 sec. These lines are also zero lines for the various curves.

his measurements to be 1.060. In the calculations of specific viscosities, and relative viscosities of the substrate, the dilution of the stock substrate solution with the enzyme solution should be taken into account in accurate determinations.

The formula, if applicable to the break down of starch with α-amylase, should give correct values if the enzyme concentration, the substrate concentration and the polymerization degree of the substrate are changed within reasonable limits.

Blom and Bak [6], whose formula is equivalent with mine at constant substrate concentration [4], have found that the time required for the viscosity to decrease from one given value to another, is inversely proportional to the enzyme concentration. They also found that starch from various plants gave the same result on assaying the activity of equally active enzyme solutions.

The influence of the concentration of the starch solution was investigated in the first report of this investigation and the results given in Table 4 there. It was shown that good values are obtained if the concentration of the starch in the reaction mixture is not higher than about $2\frac{1}{2}$ %.

The influence of the initial degree of polymerization of the starch has been further investigated. Samples of potato starch were treated for various times

with 1.5 *N* hydrochloric acid, whereupon the starch was thoroughly washed and dried. Starch solutions were prepared in the manner previously recommended [4]. The activity of a given enzyme solution was determined with these various starch solutions. The viscosity measurements expressed as $1/\eta_{sp}$ and $1/\ln \eta_r$ are given in Figs. 1 and 2 respectively, and the results from the calculations in Table 2.

In all experiments 3 ml of enzyme solution were mixed with about 30 ml of starch solution, the accurate weight determined and used for the activity calculations. The initial specific viscosity of the starch solution was calculated from measurements using a mixture of the stock solution and water in the same proportions as in the enzyme assays.

Table 2. Viscosimetric assay of the activity of an amylase solution with potato starch.

Time for the treatment with 1.5 *N* HCl	η_{sp} (initial)	Curve	$100 \cdot c_s$	Activity (μA/g)	
				using η_{sp}	using $\ln \eta_r$
0	30	*a*	2.271	3.24	3.50
		b	2.274	3.32	3.66
5 min	10	*c*	2.271	3.36	3.47
		d	2.274	3.31	3.48
21 hours	5.5	*e*	2.275	3.42	3.57
115 hours	0.8	*f*	2.276	3.40	3.52

These experiments show that potato starch may be partially depolymerized with hydrochloric acid without any appreciable influence on the results of viscosimetric amylase assays. If the assays are performed with starch which has a very high degree of polymerization, there will be some deviations from the expected straight line, indicating that neither Staudinger's nor the modified Arrhenius-Staudinger formula is completely valid here. How far the enzymatic depolymerization will follow the linear relationship between the functions of the viscosity and the time cannot accurately be judged from these experiments, since the Oswald viscosimeter in all these experiments had a flow time of only about 10 seconds for the pure solvent. In the experiments where the starch concentration was about 2.3 %, measurements where $1/\eta_{sp}$ was below 1.5 could be taken into consideration.

DILUTION OF ENZYME SOLUTIONS TO SUITABLE STRENGTHS

It is well-known that various enzyme solutions become less stable when strongly diluted. This also holds true for malt α-amylase if diluted with water. Difficulties seldom arise if the activity is to be determined when the amylase concentration must be relatively large, but if the assay is to be performed viscosimetrically, the amylase concentration must be relatively small, and the inactivation may cause errors in the results. A fairly reliable method for obtaining stable dilute enzyme solutions is to use a boiled enzyme solution for the dilution. This method gives good result but is too enzyme-consuming to be of practical use. I have tried a 1 % egg albumine solution for the dilution and always found good stability, but these solutions are a little more difficult to treat, for they give rise very easily to disturbing bubbles in the viscosimeter, which may be very difficult to remove.

Wallerstein [7] recommended the addition of 1 g of calcium sulfate to 1000 ml of water, corresponding to a 0.012 N solution. Nakamura [8] found that an addition of calcium salts corresponding to about a 0.001 N solution was necessary to give solutions of malt amylase maximum stability. Holmbergh [9] has recommended an addition of 0.4 g of calcium acetate to 100 ml of water, corresponding to a 0.05 N solution, for the extraction of malt. Kneen, Sandstedt, and Hollenbeck [10] found that an addition of 0.004 mg calcium ion per ml solution, corresponding to a 0.0002 N solution, gave a considerably increased stability.

The following experiments give an idea of the stability of malt α-amylase solutions on dilution.

From a malt extract, in which the β-amylase had been distroyed by heating to 70° C, a dry preparation of malt amylase was made by precipitation with tannin and subsequent washing with aceton and ether, whereupon the amylase was sucked off and dried. A *dialyzed* 2 % solution of this preparation in distilled water had an activity of 540 $\mu A/g$. Samples of this solution were diluted 500 times at a favourable pH with distilled water, with calcium chloride solutions of various strengths, with boiled 2 % amylase preparation solution and with boiled 2 % amylase preparation solution with addition of potassium oxalate to a final concentration of 0.01 N. The activity of the diluted solutions immediately after the mixing and after various times was determined, and the results are given in Fig. 3.

The values in Fig. 3 indicate the importance of calcium ions for the stability of the amylase. Some stabilizing agent other than calcium ion seems, however, to be present in the boiled amylase solution.

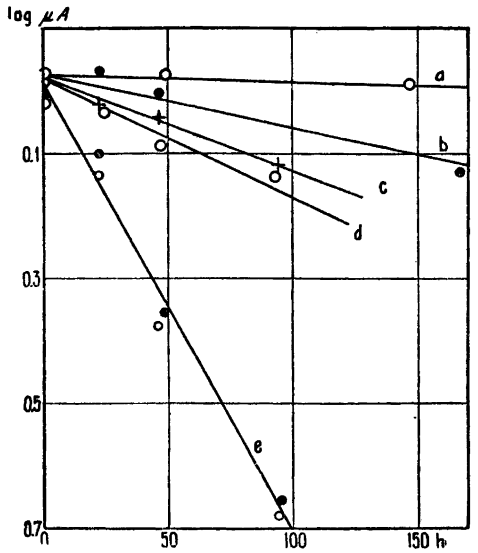


Fig. 3. The stability of diluted amylase solutions.
a (rings): boiled amylase solution, pH = 6.0
b (dots): 0.1 N $CaCl_2$ solution, pH = 6.1
c (crosses): 0.01 N $CaCl_2$ solution, pH = 6.3
d (rings): 0.0001 N $CaCl_2$ solution, pH = 6.5
e (dots): distilled water, pH = 5.9
(rings): boiled amylase solution, 0.01 N $K_2C_2O_4$, pH = 6.0

THE INFLUENCE OF THE TEMPERATURE ON THE VISCOSIMETRICAL ASSAY OF AMYLASE

It is customary to express the influence of the temperature by stating how many times faster the reaction proceeds if the temperature is raised 10° C. This can be expressed mathematically in the following way, if k_{t+10}/k_t is this factor:

$$A^{t+\Delta t^\circ} = A^{t^\circ} \cdot \left[\frac{k_{t+10}}{k_t}\right]^{\frac{\Delta t}{10}} \tag{7}$$

Investigations on the activity of amylase at various temperatures have already been performed long time ago by Roberts [11] and by Vernon [12].

Roberts investigated the activity of pancreatic diastase at temperatures from 3 to 70 °C. He found an activity maximum at 30—45°, which probably depends on the irreversible heat inactivation of the enzyme. From his activity values at 3—5, 10, 15, and 20 ° I have calculated the increase in the reaction velocity and found the factor to be 2.1 for 10 degrees.

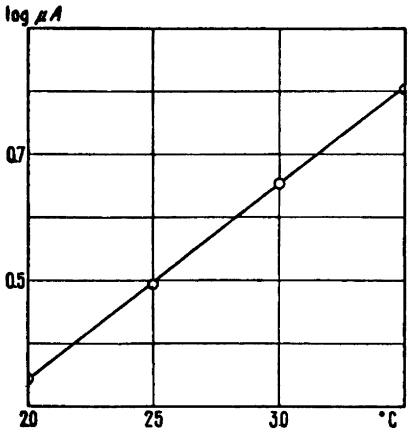


Fig. 4. The activity of an amylase solution as a function of the temperature.

Vernon gives in a figure the results of activity determinations at temperatures from 20 to 60° C of pancreatic diastase in a 0.2 % NaCl solution. If the logarithms of his activity determinations are plotted against the temperature, the points lie tolerably well on a parabola. From the inclination of its tangent at 25° C, a factor can easily be obtained, giving the increase of the reaction velocity for a temperature increase of 10° and valid at least for the first part of the curve, *i. e.* 20—30° C. In this way I have calculated the factor from Vernon's measurements and found the value 2.05.

v. Euler and Svanberg [13] also calculated their value for this factor from Vernon's measurements and gave the value 2.0.

The present author has performed some experiments in order to ascertain the influence of the temperature on the activity of amylase and the influence of errors in the temperature on the result.

The activity of an amylase solution was determined at 20, 25, 30, and 35 °C. For the calculations of the specific viscosities the respective values of the viscosity of the buffer solution were employed. The logarithms of the activity values are plotted against the temperature in Fig. 4, and from the inclination of the straight line through the points the value of the factor was calculated to be 2.03.

An estimation of the influence on the result of errors in the temperature can be obtained in the following way.

The value of the enzymic activity is proportional to the derivative $d\frac{1}{\eta_{sp}}/dt$, where

$$\frac{1}{\eta_{sp}} = \frac{\eta_{solvent}}{\eta_{solution} - \eta_{solvent}}$$

An error in the temperature determination will give raise to the use of an incorrect value for $\eta_{solvent}$ (in the calculations: an incorrect value for the corrected flow time for the pure solvent). In the case of the difference $\eta_{solution} - \eta_{solvent}$ this error may be neglectible, if the flow time of the solution is much longer than the flow time of the solvent.

If A = the activity calculated from measurements at $t + \Delta t$ °C under the assumption that the temperature was $t°$, and if the viscosity of water at $t°$ is denoted by η_t, we have

$$A^{t+\Delta t°} = A \cdot \frac{\eta_t + \Delta t}{\eta_t}$$

$$A^{t°} = A \cdot \frac{\eta_t + \Delta t}{\eta_t} \cdot \left[\frac{k_{t+10}}{k_t}\right]^{-\frac{\Delta t}{10}}$$

At 30° C and $\Delta t = 1$, we get

$$A^{30°} = A \cdot \frac{0.7840}{0.8007} \cdot 2^{-0.1} = A \cdot 0.98 \cdot 0.93 = A \cdot 0.91$$

An error of 1 degree in the temperature thus gives raise to an error in the result of about 10 %. Most of this error (about 7 %) originates from the increased velocity of the break down, and is consequently present in all methods for amylase assay. If the error originating from imperfect temperature determination must not exceed 1 %, the temperature assay should be accurate to within 0.1° C.

COMPARISON OF THE AMYLOLYTIC ACTIVITY OF α-AMYLASE AND ITS POWER TO LIBERATE REDUCING SUGARS

The activity of an amylase solution was determined viscosimetrically. Its power to liberate reducing sugars was determined by Blom and Rosted's [14] modification of Linderstrøm-Lang and Holter's [15] method. The activity was calculated in $\mu A/g$ and in mg maltose per minute and milliliter solution. The results are given in Table 3.

Table 3. The activity of an amylase solution in $\mu A/g$ and in mg maltose/min · ml.

$\mu A/g$	mg maltose / min · ml
1680	28.8
1690	29.0
1690	29.0
1620	30.2
	30.2

From this table we gather that the amylase amount 1 μA liberates reducing sugars equivalent to 0.018 mg maltose per minute.

SUMMARY

1. The calculation of enzyme activities from viscosimetric measurements are simplified by means of a specially calculated table, giving — for various concentrations and amounts of stock substrate solution — the factor, by which the derivative $d\frac{1}{\eta_{sp}}/dt$ or $d\frac{1}{\ln \eta_r}/dt$ must be multiplied to give the activity of the enzyme solution.

2. Certain deviations from the expected viscosity of starch solutions may occur in the beginning of the break down, when the degree of polymerization is still very high.

3. Enzyme solutions, which are too strong to be measured without previous dilution, should be diluted with a 0.1—0.01 N calcium salt solution.

4. The velocity of the break down increases 2.03 times if the temperature is raised 10° C.

5. In viscosimetric determinations of amylase activity, an error in the temperature assay of 0.1° C gives raise to an error in the activity of 1 %.

6. One conversion factor is calculated and tentatively suggested: the amylase activity 1 $\mu A/g$ corresponds to the power to liberate reducing sugars equivalent to 0.018 mg maltose/min · ml.

For this investigation the author received financial support from *Statens Tekniska Forskningsråd* and *Statens Naturvetenskapliga Forskningsråd*. Prof. Karl Myrbäck kindly granted me the use of his laboratories. Miss Anna Hörnfeldt, Miss Helena Bischof and Miss Margareta Kjellén assisted in performing the experiments. The English translation was revised by Mrs William Cameron. For all this help I wish to express my cordial thanks.

REFERENCES

1. Hultin, E. *Svensk Kem. Tid.* **58** (1946) 281.
2. Hultin, E. *Acta Chem. Scand.* **3** (1949) 625.
3. Hultin, E. *Svensk Kem. Tid.* **60** (1948) 40.
4. Hultin, E. *Acta Chem. Scand.* **1** (1947) 269.
5. Reyher, R. *Z. physik. Chem.* **2** (1888) 744.
6. Blom, J., and Bak, A. *Z. physiol. Chem.* **256** (1938) 197.
7. Wallerstein, M. U. S. 905 029 (1908).
8. Nakamura, H. *J. Soc. Chem. Ind. Japan* **34** (1931) 265 B.
9. Holmbergh, O. *Arkiv Kemi Geol. Mineral.* **A 11** (1935) no. 20.
10. Kneen, E., Sandstedt, R. M., and Hollenbeck, C. M. *Cereal Chem.* **20** (1943) 399.
11. Roberts, W. *Proc. Roy. Soc. London* **32** (1881) 145.
12. Vernon, H. M. *J. Physiol.* **27** (1901) 174.
13. Euler, H. v., and Svanberg, O. *Z. physiol. Chem.* **112** (1921) 193.
14. Blom, J., and Rosted, C. O. *Acta Chem. Scand.* **1** (1947) 32.
15. Linderstrøm-Lang, K., and Holter, H. *Medd. Carlsberg Lab.* **19** (1933) no. 14.

Received June 13, 1949.