Communications to the Editor

Comparison of α-Amylase Activities from Different Assay Methods

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Accepted for publication June 23, 1986

α-Amylase enzymes (1,4-α-D-glucanohydrolase, E.C.3.2.1.1) catalyze the hydrolysis of α-1,4 glucosidic linkages in polysaccharides of three or more α-1, 4-linked D-glucose units to produce maltose and larger oligosaccharides.[1,2] Since there are many different assay methods and definitions for a unit of α-amylase enzyme activity, it is almost impossible to compare enzyme activities. One reason is that most groups working with α-amylase developed their own enzyme assay systems, each with its own unit of activity.[3]

The objective of this communication is to provide a simple relationship among α-amylase activities, which allows comparison of the enzyme activities in the literature. Even though the assay methods and definitions of an enzyme unit are different, enzyme activities can be correlated as a function of incubation temperature, incubation time, dilution factor, and measurement methods. This result will be useful in finding a microorganism or culture conditions which give the highest enzyme activity.

VARIOUS ASSAY TECHNIQUES

Amylase action is characterized by simultaneous changes of the following properties of the substrates: 1) decrease of viscosity, 2) increase of reducing power, 3) change in iodine color reaction, 4) change in optical rotatory power, and 5) decrease in the turbidity of glycogen solution.[4] Most of the reports are based upon one of the following methods for detecting α-amylase activities on starch:[5] 1) the release of reducing sugars from a starch substrate is measured;[6] 2) the decrease of the specific reaction between iodine and residual starch is measured;[7] and 3) a chromogenic group is attached to the substrate and the release of this substrate into the soluble fraction is monitored by a change in optical density.[8,9] Amylose was also suggested as a substrate.[10] The methods using low-molecular-weight substrate with a defined structure such as maltotetraose and maltoheptaose have been recommended in recent years, but these methods were developed and tested primarily for clinical applications.[11–13]

The Nelson colorimetric copper method[14] was found to give more accurate results in measuring reducing sugars over a method using alkaline 3,5-dinitrosalicylate.[15] The Nelson colorimetric copper method gives identical reducing values for equimolar reducing of maltodextrins; the measurement of the apparent maltose produced in an amylase reaction was directly proportional to the amount of enzyme present.[16] Many improvements in the α-amylase assay technique have been made to get more accurate values of the enzyme activities. However, no attempt has been made to correlate each method and its corresponding results.

Usually, enzymes are assayed based on their reaction with the substrate under test conditions. Test conditions include incubation time, incubation temperature, pH, and, sometimes, calcium ion concentration for thermal stability. After reaction with the substrate, the unreacted substrate concentration is measured using an iodine method or the product concentration is measured using a reducing sugar method to find the extent of reaction by the α-amylase. One IU (international unit), a commonly accepted unit of measure, is defined as the amount of enzyme that catalyzes the conversion of 1 μM substrate/min under standardized conditions of substrate concentration, optimum pH, absence of inhibitors, and presence of activators.[17] Even

Biotechnology and Bioengineering, Vol. XXX, Pp. 147–151 (1987)

CCC 0006-3592/87/010147-05$04.00

Table I. Examples of typial α-amylase assay methods.

Method	Description	Definition of unit	Test conditions	Reference
Wohlgemuth (iodine)	measure the time for attaining the definite iodine coloration	mL starch (1.0%) hydrolyzed by 1 mL enzyme	40–60°C, 30 min	24,25
Fisher and Stein (reducing value)	measure the reducing group by the dinitrosalicyclic acid procedure	1 mg of reducing sugar as maltose released from 1.0% starch solution	65°C, 3 min	26,27,21
Bird and Hopkins (iodine)	measure the decrease in extinction of the starch–iodine color at 620 nm	1 unit will liberate 1.0 mg maltose from starch	25°C, 10 min	7,28
Insoluble dyed amylose	release of chromogen-bound oligosaccharide from insoluble dyed amylose	1 absorbance unit corresponds to 0.06 IU/mL α-amylase	37°C, 30 min	29
Fuwa (iodine)	amylose (0.2%) is used as a substrate	the amount of amylose (mg) which decreased 10% of the blue value (700 nm OD)	37°C, 30 min	10,30
SKB (iodine)		the amount of enzyme which degrades 5.26 mg of starch to a certain iodine value	37°C, 1 h	31

enzyme activities expressed as international units are seldom found in an α-amylase system. Table I shows examples of typical α-amylase assay methods. It is impossible to compare the results from different methods because of the various incubation conditions and different definitions of enzyme activity units. The differences in various assay methods are incubation time, incubation temperature, and measurement method.

α-Amylase was found to decrease the iodine color very rapidly and to increase the reducing values very slowly.[18] The differences between the rate of decrease in blue iodine color and the rate of increase in reducing value was due to the multiple attack mechanism of the α-amylase enzyme.[19] The change of the reducing value is ca. 1/3–1/6 of the iodine color change, depending on the extent of reaction for *B. amyloliquefaciens* and *A. oryzae*.[16,20]

In this communication, the study of the effects of incubation time and incubation temperature on enzyme activity is emphasized to correlate enzyme activities for the starch–iodine or reducing sugar value methods. Also, results from different measurement methods were compared for the enzyme under study.

MATERIALS AND METHODS

The starch–iodine method[7] was used as a reference assay method.

Preparation of Chemicals

A soluble potato starch (Fisher Scientific) solution was prepared to give 20 mg starch/mL. The starch solution was diluted 1:1 with a 0.04*M* phosphate buffer at pH 5.9. The working iodine reagent was prepared fresh by diluting 1.0 mL of stock solution (500 mg iodine and 5.0 g potassium iodide/100 mL water) 100 times. *Bacillus amyloliquefaciens* α-amylase (Sigma Chemical) and Taka-therm L-170 α-amylase of *Bacillus licheniformis* (Miles Laboratory) were diluted and used for the enzyme assay. All the chemicals used were reagent grades.

Assay Procedure

Five milliliters of substrate solution is added to a test tube and maintained for 10 min at an incubation temperature in a water bath. Enzyme (0.5 mL) is added to the substrate solution and incubated under the test conditions. The digest is added to 5 mL stopping reagent (0.1*M* HCl). After mixing, 0.5 mL of this mixture is added to 5.0 mL working iodine solution. The intensity of blue color is measured in a colorimeter (Klett

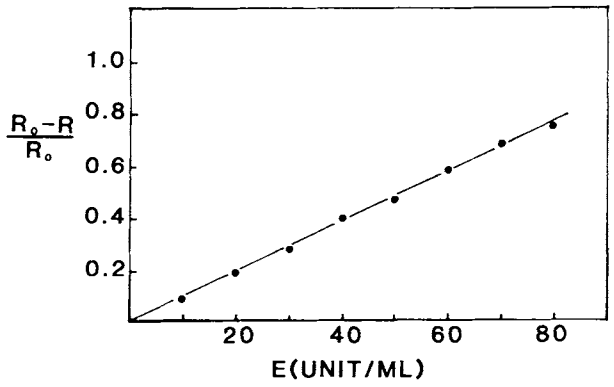

Figure 1. Effect of dilution rate in the enzyme assay. Samples were prepared by diluting an enzyme solution using a buffer solution.

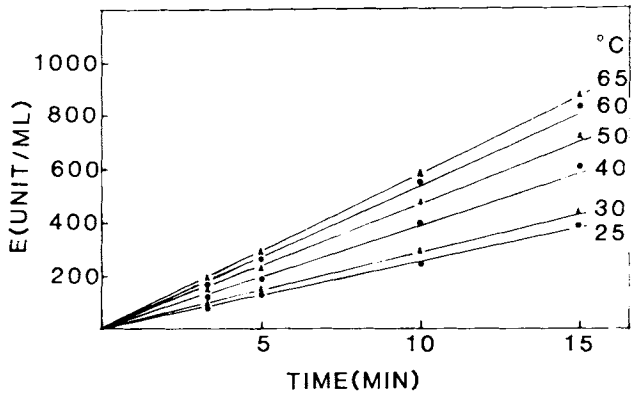

Figure 2. Effect of incubation time and temperature upon the activity of the *B. amyloliquefaciens* α-amylase.

Summerson, Klett Manufacturing Co.) using a red filter. The instrument is set to zero with an iodine blank containing neither enzyme nor substrate. The activity of the enzyme is calculated from the formula,[6]

$$\text{Activity (unit/mL)} = D\,[(R_0 - R)/R_0] \times 100 \quad (1)$$

where R_0 is the absorbance of the substrate–iodine complex in the absence of enzyme; R is the absorbance of the digest; and D is the dilution factor of the enzyme. The enzyme solution was diluted when necessary so that the ratio $(R_0 - R)/R_0$ was between 0.2 and 0.7.

RESULTS AND DISCUSSION

Effect of Dilution

An enzyme solution was diluted with buffer and assayed. The result which can be seen from Figure 1 shows that $(R_0 - R)/R_0$ is proportional to E (unit/mL). However, the activities of the enzyme calculated from eq. (1) were the same at different dilutions. Thus, there is no effect of dilution on determining the enzyme activity from eq. (1).

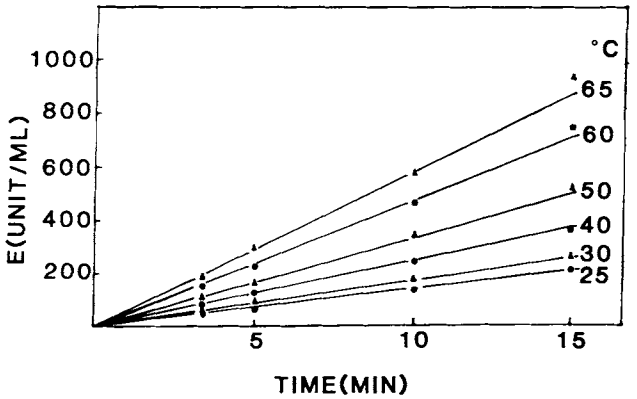

Figure 3. Effect of incubation time and temperature upon the activity of the *B. licheniformis* (Taka-therm) α-amylase.

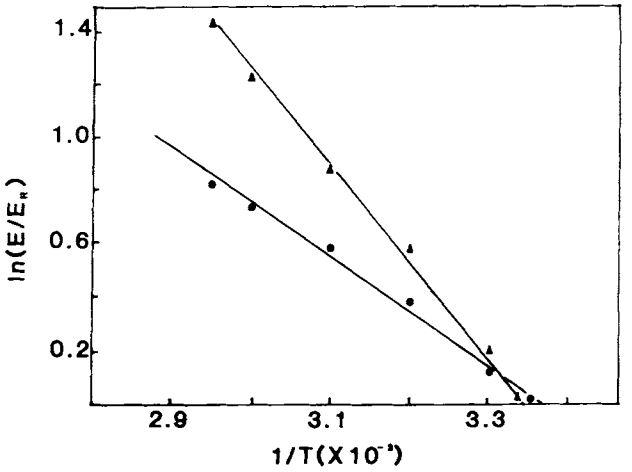

Figure 4. Arrhenius plots of the activity of an α-amylase; E_R is a reference activity (25°C, 10 min): (●) *B. amyloliquefaciens* enzyme and (▲) *B. licheniformis* enzyme.

Effect of Incubation Time and Temperature

Figure 2 shows the results with *B. amyloliquefaciens* α-amylase while Figure 3 shows the results with Taka-therm α-amylase. A linearity was observed between enzyme activity determined from eq. (1) and incubation time up to 30 min for various temperatures (from 25 to 65°C). A weighted average method was used to calculate the relationship between the activity and the incubation time. Also, the apparent activation energies ΔE_{app} $(= \Delta E'_{app}/R)$ for the enzyme systems were calculated from the slope in Figure 4.

From the plots of Figures 2–4, ΔE_{app} for *B. amyloliquefaciens* α-amylase was 2020 K and ΔE_{app} for *B. licheniformis* (Taka-therm) α-amylase was 3620 K. The main reason for the differences in the activation energy is due to different structures of the enzymes and the different thermal stabilities of the enzymes.[21]

These characteristics differ from one strain to another even in the same *B. amyloliquefaciens*.[21] From the above results, a normalized enzyme activity based

Table II. Enzyme activities from different measurement methods.

Sample	A	B	C	D	E
1) Enzyme activities (iodine method)[a]	19	36	52	65	78
2) Enzyme activities[b] (reducing value)[c]	4.7	6.4	9.4	10.7	12.2
Conversion factor (sample 1/sample 2)	4.0	5.6	5.5	6.1	6.4

[a] One enzyme unit is defined as the amount of enzyme that 1 mg of starch (1%) hydrolyzed by 1 mL of enzyme in 10 min at 25°C and pH 5.9 (unit/mL).

[b] One enzyme unit from the reducing sugar value method can release 1 mg of reducing sugar (as maltose) from 1% starch solution in 10 min at 25°C and pH 5.9 (unit/mL).

[c] This was measured using the Nelson copper method.

Table III. Comparison of enzyme activities.

Assay method	Test conditions	Reported activity[a] (unit/mL)	Normalized activity[b] (unit/mL)	Microorganism	Reference
Wohlgemuth	40°C, 30 min	5000	1208	*B. subtilis*	25
Fisher and Stein	65°C, 3 min	362	2700[d]	*B. stearothermophilus*	27
Fuwa	37°C	2200	1015	*B. amylolique-faciens*	32
Fuwa	37°C, 30 min	1.5×10^4[c]	769	Immobilized *B. subtilis*	30
Wohlgemuth	60°C, 30 min	1000	164	*B. subtilis*	33

[a] This is the activity defined in the literature (see Table 1)
[b] This is the activity normalized at 25°C and 10 min. as iodine method according to the method developed in this report
[c] The 5 DP (dextrinizing power) units/mL in Fuwa's method can produce ca. 1 mg/mL reducing sugar.
[d] The conversion factor of 5 was used as an approximation.

on a reference test condition, E_R (25°C, 10 min), can be obtained as follow:

$$E_R = E\left(\frac{t_R}{t}\right)e^{-\Delta E_{app}\left(\frac{1}{T_R}-\frac{1}{T}\right)} = D\frac{R_0-R}{R_0}\left(\frac{t_R}{t}\right)e^{-\Delta E_{app}\left(\frac{1}{T_R}-\frac{1}{T}\right)} \quad (2)$$

where t_R = 10 min; T_R = 298 K; and E is a value determined at T (K) and optimum pH for t min. The normalized enzyme activity, E_R, at the reference condition will be used in comparing the results in the literature.

Comparison of Measurement Methods

Enzyme activities were obtained using the starch–iodine method and the reducing value method for the enzyme from *B. amyloliquefaciens* as shown in Table II. The enzyme activities from the iodine method were ca. 4–6 times higher than those from the reducing value method depending on the extent of reaction. This result agrees well with the data in the literature.[16]

Correlation of Enzyme Activities

The dependences of incubation temperature and incubation time were obtained using the starch–iodine method. This result can also be used for the reducing sugar value method, since the reducing value is also proportional to the amount of enzyme.[16] The results from the reducing value method can be converted into equivalent results from the iodine method by multiplying by a conversion factor of 4–6. It is impossible to compare the enzyme activities accurately from the two different measurement methods because the extent of reaction for the enzyme assay is not reported.

Table III shows an application of the above results which allows comparison of enzyme activities with published results. The results from the *B. amyloliquefaciens* α-amylase can be compared with the results from the *B. subtilis* α-amylase because of their similarities.[22,23] From Table III, it is easy to compare the results of the α-amylase activities and find a microorganism or culture conditions which give the highest enzyme activity.

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