Journal of Industrial Microbiology, 5 (1990) 295–302
Elsevier



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# A simple and reliable turbidimetric and kinetic assay for alpha-amylase that is readily applied to culture supernatants and cell extracts

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Received 9 June 1989
Accepted 18 July 1989

*Key words:* Alpha-amylase assay; Turbidity; Kinetic determination

## SUMMARY

A simple, reliable and sensitive assay for alpha-amylase activity is reported, together with its theoretical derivation, that overcomes many of the problems encountered with other assays, especially when attempting to assay alpha-amylase activity in crude cell extracts or culture supernatants. The method relies on the reduction in turbidity that occurs upon digestion of a starch suspension with alpha-amylase. The initial rate of decrease in turbidity is shown to be proportional to a wide range of enzyme concentrations, permitting a rapid spectrophotometric and kinetic determination of alpha-amylase activity.

## INTRODUCTION

Alpha-amylases (1,4-alpha-D-glucan-4-glucanohydrolase, E.C.3.2.1.1) are endoglucanases widely distributed among animals, plants, fungi and bacteria. They are believed to cleave at random within polysaccharide chains consisting of alpha-1,4-linked glucose residues, producing initially oligosaccharides of various lengths and, in some cases, maltose and glucose as final products [5]. The molecular complexity of their substrates, the varying modes of hydrolysis adopted by different alpha-amylases [13] and the frequent contamination of samples with exoglucanases have served to complicate the quantitation and comparison of enzymes from different sources and a simple and reliable assay has yet to be found. Alpha-amylase activity is most frequently assayed by measuring the release of reducing sugar from a suitable polymeric substrate using the subsequent reduction of copper sulphate [19] or dino-

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trosalicylic acid [1,3] to determine, spectrophotometrically, the level of enzyme activity. A commonly used alternative involves the release of a chromogenic aglycone from nitrophenyl alpha-malto-oligosaccharides [2,7,17].

All of these methods lack sensitivity, predominantly because they fail to make allowance for the endoglucanolytic mechanism of alpha-amylases. Many of the cleavages that occur during these assays are not detected; the first method suffers from the weak reducing power of large oligosaccharides, and the second from the inevitability that many cleavages will not lead to release of the chromophore. Furthermore, since these assays do not discriminate between endoglucanases and exoglucanases, the presence of a low level of, for example, glucoamylase in a sample will severely reduce the ability to measure true levels of alpha-amylase activity. This is a particularly difficult problem when attempting to assay alpha-amylase activity in crude cell extracts or culture supernatants.

Assays that are more specific for endoglucanases have been developed. The sensitivity of the iodine binding method [4,18] is still low, and it suffers, at least when using unpurified samples, from interference by proteins [14]. Dyes such as Cibacron Blue [10], Remazol Blue [16], Reacton Red [20] or Procion Yellow [8] have been covalently coupled to starch and alpha-amylase activity determined by measuring, spectrophotometrically, the release of solubilised dye. While potentially providing better estimates of alpha-amylase activity, these assays are time-consuming and there is some difficulty in obtaining independent batches of modified starch that possess the same degree of dye substitution. Since they rely almost exclusively on endoglucanolytic cleavage, measurements of the rate of decrease in the turbidity or viscosity of a starch suspension in the presence of alpha-amylase appear to be the most sensitive and reliable methods to assay alpha-amylase activity. However, the convenient and accurate measurement of changes in viscosity requires the use of sensitive, sophisticated and expensive apparatus. Although a turbidimetric method was developed by Waldron [22] and used by several authors to assay alpha-amylase activity in a variety of physiological samples [6,15,23], the method was not used outside of the clinical field and appears to have been abandoned. Furthermore, the assay lacked the credibility of a theoretical derivation.

In this paper we describe a simple, reliable and sensitive assay that correlates alpha-amylase activity with changes in turbidity of a starch suspension. We provide a theoretical basis for the method and then demonstrate its application. The decrease in turbidity is linear with time for the first 0.1 $OD_{300nm}$ unit for a range of enzyme concentrations. In addition, the rate of decrease in turbidity is proportional to a wide range of alpha-amylase concentrations. Such characteristics permit the derivation of a calibration curve, using a dilution series of a commercially purified alpha-amylase of known concentration, which can then be used to estimate the alpha-amylase activity of a sample of interest; alternatively, the assay can be used to compare relative enzyme activities using arbitrarily defined units of activity.

## MATERIALS AND METHODS

*Substrate and enzymes*

A 1% (w/v) potato starch (BDH) suspension in 50 mM $K_2HPO_4$-$KH_2PO_4$ pH 6.8, 50 mM KCl buffer was boiled for 10 min with vigorous stirring and autoclaved for 15 min at 120°C; it gave a reproducible starting $OD_{300nm}$ of $0.67 \pm 0.02$. This suspension can be kept for at least a week at room temperature without undergoing any significant retrogradation as monitored by a change in optical density. (Starch suspensions prepared from maize and acid-hydrolysed starches underwent rapid retrogradation and were not used.) Alpha-amylase from *Bacillus amyloliquefaciens* (Sigma) was used to establish a calibration curve. One unit of enzyme activity was defined by the supplier as the amount of enzyme that will liberate 1 mg of maltose from starch in 3 min at pH 6.8 in 50 mM $K_2HPO_4$-$KH_2PO_4$, 50 mM KCl buffer at 20°C. Appropriate dilutions were made in the phosphate buffer described above and the samples stored at room temperature.

*Assay procedure*

900 $\mu$l of the 1% (w/v) potato starch suspension were mixed thoroughly with 100 $\mu$l of the enzyme solution in a cuvette and the rate of decrease of $OD_{300nm}$ over the first 0.1 $OD_{300nm}$ unit was recorded using a Pye Unicam SP1700 Ultraviolet Spectrophotometer. Controls were carried out using 100 $\mu$l of phosphate buffer instead of enzyme solution and did not reveal a significant decrease of $OD_{300nm}$.

## THEORETICAL DERIVATION

The gelatinised starch suspension, prepared as described in Materials and Methods, contains aggregates of amylose molecules. The turbidity of the sample will be determined by the size and concentration of these aggregates. For a sample of path length $l$, the turbidity $\tau$ is given by Kerher [9] as:

$$I = I_o \exp(-\tau l)$$

where $I$ and $I_o$ are the intensities of the transmitted and incident light respectively. For particles whose refractive index is close to that of the suspending medium ($n_o$) and whose major size dimension is larger than $\lambda/10$, where $\lambda$ is the vacuum wavelength of the incident light, then the turbidity will be given by Koch [11] as:

$$\tau = \frac{16\pi^2}{3}\left(\frac{\partial n}{\partial c}\right)^2\left(\frac{q}{n_o}\right)^2\frac{v}{(\lambda')^4}Q = A_q{}^2 \quad \text{(eqn. 1)}$$

where $\left(\frac{\partial n}{\partial c}\right)$ is the specific refractive index increment, $q$ the anhydrous mass of the particle, $v$ the number concentration of particles, $\lambda'$ the wavelength of the light in the medium and $Q$ a factor which takes into account the angular distribution of the scattered light by integrating the particle scattering function over all scattering angles. During the course of the enzymatic reaction $A$ is a constant and only $q$ decreases; hence the clarity of the sample increases because of digestion of the aggregates and the release of small fragments which do not contribute significantly to the measured turbidity. During the early stages of the digestion process, it is reasonable to assume that the enzyme attacks all particles equally and that the decrease in turbidity is dominated by the decrease $\Delta q$ in anhydrous mass of each aggregate. Provided $\Delta q \ll q$ then the decrease in turbidity $\Delta\tau$ can be shown to be $\Delta\tau \simeq 2\Delta q A q$. The total mass $q$ of the aggregate will be related to the number of glycoside bonds ($N + N_A$), where $N_A$ is the fraction of such bonds initially freely available for enzymatic attack. Thus:

$$q = q_m (N + N_A)$$

where $q_m$ is the anhydrous mass of a monomer unit. Clearly, during the early stages of enzymatic attack the change in mass will depend on the loss of available sites $\Delta N_A$. Hence:

$$\Delta\tau \simeq 2Aq\, q_m\, \Delta N_A$$

In the early stages of digestion, where the change in the number of the available sites for enzymatic attack is small, the concentrations of enzyme ($C_e$) will be proportional to the number of available sites at time zero and the decay process will be approximately first order with:

$$N_A(t) = N_A \exp(-K_1 C_e t) \quad \text{(eqn. 2)}$$

where $K_1$ is a constant.

In the initial stages of digestion:

$$\underset{t\to 0}{\text{Limit}}\left\{\frac{\partial N_A(t)}{\partial t}\right\} = \frac{\Delta N_A}{\Delta t} = -K_1 C_e$$

The concentration $C_e$ can be related to the total enzyme concentration ($C$) by considering the interactions:

$$\text{(available site)} + \text{enzyme} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \text{(enzyme + site)}$$

If all sites are considered to be identical with a probability of absorption of $\sigma$ then:

$$\frac{\varphi}{1\varphi} = K_1 C$$

and since $C_e = K_2\varphi$ where $K_2$ is a constant, then:

$$C_e = \frac{K_2K_1C}{1 + K_1C}$$

Clearly the initial slope of a plot of turbidity against time $\left(\frac{\Delta\tau}{\Delta t}\right)$ can be related to the enzyme concentration:

$$\frac{\Delta\tau}{\Delta t} = (2Aq\ q_m - K_1)\left(\frac{K_2K_1C}{1 + K_1C}\right)$$

At low enzyme concentrations:

$$\frac{\Delta\tau}{\Delta t} = 2Aq\ q_m - K_1K_2K_1C$$

and will be proportional to the enzyme concentration. Such a linear relationship is shown in Fig. 1. At higher enzyme concentrations the available sites will become saturated and the rate of decrease of turbidity will become insensitive to further increases in enzyme concentration, i.e.

$$\frac{\Delta\tau}{\Delta t} = 2Aq\ q_m - K_1K_2$$

Evidence for a non-linear dependence at higher enzyme concentrations is shown in Fig. 2. In such circumstances the enzyme solution can be diluted until the concentration falls within the linear region.

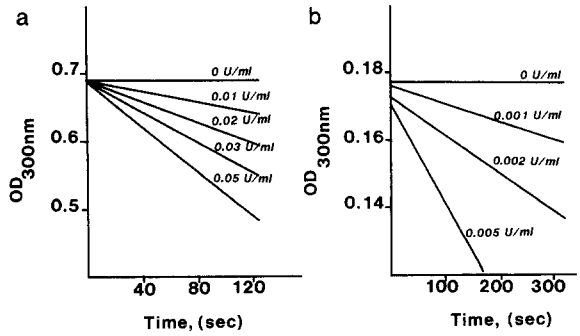


Fig. 1. a and b: $OD_{300nm}$ of a 1% (w/v) potato starch suspension plotted against time of incubation for a range of concentrations of *B. amyloliquefaciens* alpha-amylase.

## RESULTS

Since turbidity $\tau$ varies as a function of $(\lambda')^{-4}$ (equation 1), a short wavelength (300 nm, rather than the longer wavelengths generally used to measure turbidity) was used for this assay. Fig. 1 illustrates the linear relationship between decrease in turbidity and time for a range of alpha-amylase concentrations. To establish a standard curve relating the rate of decrease of turbidity to enzyme concentration the initial rate of decrease of turbidity was measured three times for each enzyme concentration and a mean value plotted against enzyme concentration (Fig. 2). For each enzyme concentration the % error of the mean value was $<10\%$. The limit of linearity of the standard curve is $<0.1$ U/ml. At enzyme concentrations $\geqslant 0.1$ U/ml the curve flattens out, as predicted by equation 1. At high enzyme concentrations ($\geqslant 0.05$ U/ml) it was important to ensure that the assay was set up rapidly enough to accurately register the decrease of the first 0.1 $OD_{300nm}$ unit. For enzyme concentrations $\geqslant 0.1$ U/ml it was necessary to dilute the sample in order to operate in the linear part of the standard curve to obtain accurate estimates of enzyme concentration.The standard curve has a slope of 2.0 $OD_{300nm}$ unit/min/U/ml alpha-amylase and the limit of sensitivity of the assay is 0.001 U/ml.

## CONCLUSIONS

The turbidimetric assay presented here has several advantages over the methods mentioned previously. It is sensitive and rapid—an assay for alpha-amylase present at levels as low as 0.001 U/ml can be carried out in 5 min. In contrast, with the same level of enzyme activity, an incubation of approximately 5 h would be required to generate detectable amounts of reducing sugar using conventional reducing sugars assays. Another advantage of the method is that it is a one-step kinetic assay; it is set up in a cuvette at room temperature and the rate of decrease of turbidity graphically recorded using a spectrophotometer. The last important practical advantage of the present method is its applicability

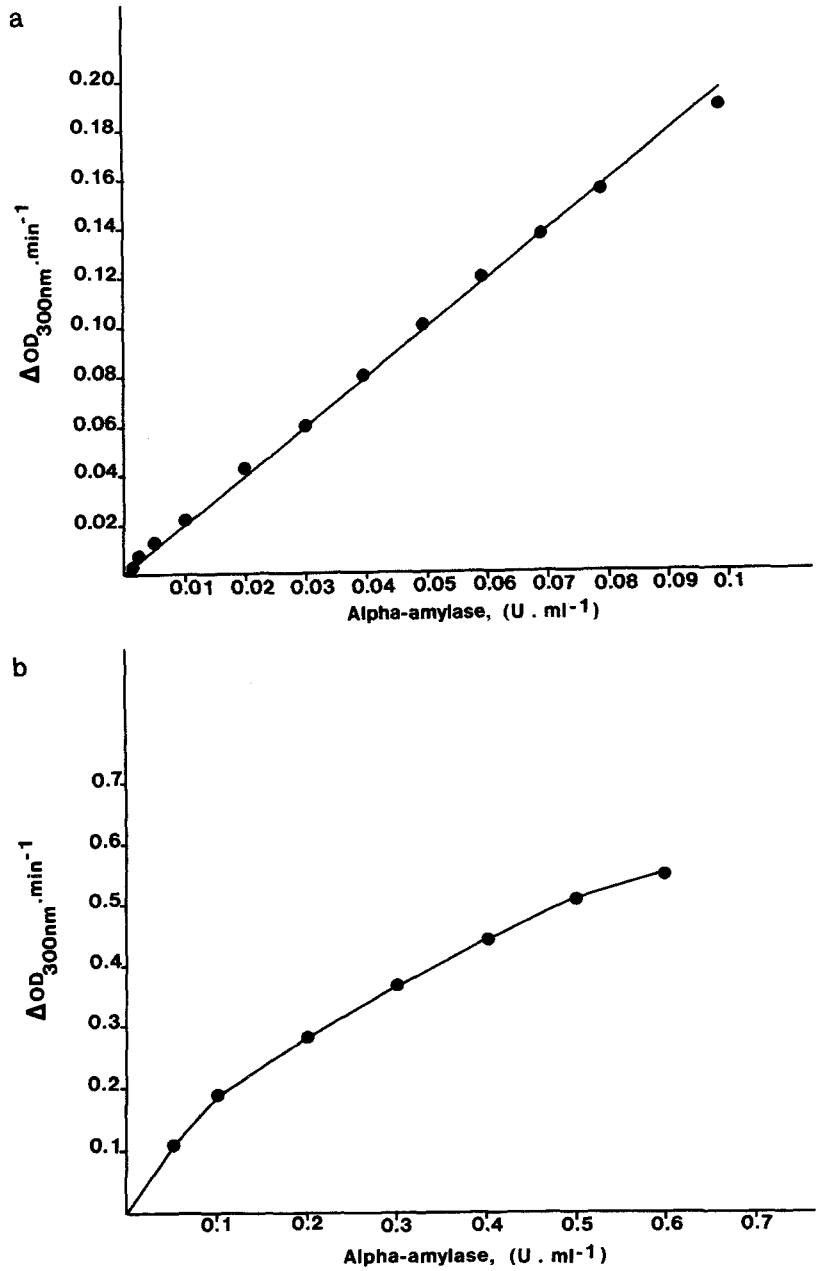


Fig. 2. a and b: Rate of decrease in $OD_{300nm}$ of a 1% (w/v) potato starch suspension plotted against concentration of *B. amyloliquefaciens* alpha-amylase. a, 0.01 to 0.1 U/ml alpha-amylase; b, 0.1 to 0.6 U/ml alpha-amylase.

to crude preparations (e.g. culture supernatants). Although the addition of such samples may contribute to the initial turbidity it will not alter the initial rate of decay. For reliability, conventional reducing sugar assays frequently require extensively purified samples; culture supernatants, for example, cannot generally be applied without some prior treatment and even after extensive dialysis background levels of reducing sugar can hinder accurate determinations.

This technique can be used in two different ways which have proved useful. A calibration curve can be established with a serial dilution of an alpha-amylase of interest and an arbitrary unit of enzyme activity can be defined (for example a unit of enzyme activity could be the amount of enzyme leading to an initial decrease of 0.1 $OD_{300nm}$ unit/min); the assay can then be used to make relative estimates of the activity of that particular enzyme. Alternatively a commercially purified enzyme of known concentration (with units of enzyme activity defined by the release of reducing sugar) can be used to establish a standard curve. The rate of decrease in turbidity of an alpha-amylase of interest can then be converted to the release of reducing sugar and enzyme activities defined in internationally recognised units. However, to do so requires that both alpha-amylases possess similar mechanisms of hydrolysis; provided that both enzymes yield the same relative activities when calibrated by both the turbidimetric and the reducing sugar methods, this ought to be possible (as was the case for the alpha-amylases of *B. amyloliquefaciens* and *Streptomyces limosus* ATCC 19778 [12]).

The turbidimetric assay described here was used to determine the effect of increasing concentrations of tendamistat, an alpha-amylase inhibitor from *Streptomyces tendae* 4158, on several purified alpha-amylases from different origins [12] and to study the regulation of the expression of the alpha-amylase gene of *S. limosus* by measuring alpha-amylase activities in crude culture supernatants [21]. We would anticipate many further applications of this technique to the estimation of alpha-amylase activity.

## ACKNOWLEDGEMENTS

We thank Steve Goulden for suggesting the turbidimetric method, Keith Chater and David Hopwood for their comments on the manucript and Merry Limberg and Anne Williams for their patient typing. This work was supported bu the Agricultural and Food Research Council via a grant-in-aid to the John Innes Institute and by direct support to the Institute of Food Research. M.-J. Virolle was supported by a Contrat Industriel pour la Formation par la Recherche (CIFRE) from the French Ministry of Industry and Research in conjunction with Roussel Uclaf.

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