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THE DETERMINATION OF BARLEY α-AMYLASE ACTIVITY

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Starch-iodine colour (S.I.C.) units of α-amylase activity are re-defined. Two improved, alternative methods of calculating results are given, either (a) by a revised graphical procedure used in conjunction with a standard graph, or (b) by a graphical procedure using points calculated from the experimental results with an empirical equation using a programmed electronic calculator. Semi-automation of the measurement of starch-iodine colours usefully reduces the work load involved in this enzyme assay. Enzyme activities may be expressed in S.I.C. units, or as a rate of increase in reducing power.

Key words: *Analysis method, α-amylase, S.I.C. units, calculations.*

Introduction

Our preferred assay for α-amylase from germinated barley is accurate, specific, and comparatively unaffected by substances that occur in grain extracts.[5] This is in contrast to the Sandstedt, Kneen and Blish assay,[8] and its variants.[1,2,3] The method is based on following the declining power of a starch substrate to give a colour with iodine as it is degraded by the α-amylase in a heat treated extract of grain.[5] The results are normally expressed in starch-iodine colour (S.I.C.) units, but these can easily be converted into increase in reducing power equivalents per unit time. Because the starch-iodine colour does not decline linearly with time it is experimentally inconvenient to attempt to determine initial reaction rates to measure enzyme activities. Instead activity is expressed as a reciprocal of the time taken for starch degradation to proceed by a fixed amount.[5] Arbitrary colour values, equivalent to experimentally determined spectrophotometric readings, are found from a standard graph.[4,5] This procedure effectively transforms the colour-time relationship, so that a straight line is produced when arbitrary colour values are plotted against experimental times for a set of samples from a digest. The time interval, t(min), between the arbitrary colour values that define the chosen extent of starch degradation is found from the graph.[5] α-Amylase activity is calculated as 100/t(min) starch-iodine colour (S.I.C.) units.[5]

The original method makes use of many spectrophotometric readings and tedious calculations.[5] Duffus[6,7] has proposed an alternative method of calculating the results but, as indicated below, the use of his method can lead to substantial errors. In this paper we report a partial automation of the method, and two improved, alternative methods for calculating the results.

Materials and Methods

The materials and techniques used were as described previously,[5] except for the points indicated below. The revised methods for calculating the results are explained in the text.

The Starch Substrate Solution.—The quantity of soluble starch (E. Merck A.G.) used to prepare the substrate solution was varied from 1·010 g/100 ml to obtain an iodine colour with the starch blank in the range E_{1cm}565nm = 0·98–1·02.

Pipettes.—Hand-held automatic pipettes (Eppendorf; Gilson; Finnpipette), with disposable plastic tips were used wherever possible. Before use performance (within 1% of notional volume) was checked by weighing successive deliveries of distilled water.

Enzyme Extraction.—The ratio of calcium acetate solution to barley was never less than 1·0 ml/grain, or 1·0 ml/35 mg. flour during enzyme extraction. Timing the 20 min extraction and heat inactivation period began when a thermometer in a tube of water, which was added to the water bath simultaneously with the tubes containing the extraction samples, registered 70°C.

The Semi-automated Measurement of Starch-iodine Colour.—Test tubes containing iodine reagent (10·0 ml) well mixed with sample (0·100 ml) were placed in sequence in the rotating rack of a sampling table, which had been made in the Departmental workshop. A probe of stainless steel hypodermic tubing descended into each tube in turn, and remained for a preselected period, generally *ca.* 2 min. A peristaltic pump induced liquid to flow from the probe via Portex tubing (1 mm i.d.) through a flow-through cell (1 cm light path, rectangular cross section, volume *ca.* 1 ml) and to waste. The flow-through cell was in the sample beam of a Beckman DB recording spectrophotometer. A cell containing an iodine reagent–water blank was in the reference beam. Optical densities were traced on the chart of a flat-bed recorder. Pumping rates and the duration of the sampling periods were so adjusted that each spectrophotometric reading reached a steady value before the sample was changed.

Results and Discussion

Semi-automation of the Determination of Starch-iodine Colour.—Samples, mixed with iodine reagent, were taken automatically and the optical densities of the solutions were measured during their passage through a split-beam recording spectrophotometer. The trace automatically provided a permanent record of the results. This system worked unattended while other manipulations were being carried out, saving a substantial amount of time.

Duffus' Method of Calculating Enzyme Activities.—Duffus proposed the use of an equation for a first order reaction in a simplified method for calculating enzyme activities.[6,7] However, for a first-order reaction colour values would decline logarithmically with time, and a semi-logarithmic plot of corrected spectrophotometric readings against time would therefore be linear. In fact this requirement is not met, as is seen when such a graph is drawn (Fig. 1). The curve is biphasic, having two straight segments joined by a curve. Thus, except within a narrow range, Duffus' method of calculation is invalid, and its use can lead to substantial errors. Furthermore in his publications Duffus[6,7] appears to confuse the use of two different methods for assaying α-amylase activity.[3,5] In fact only the more recent of these is acceptable.[5]

Re-definition of S.I.C. Units, and a Revised Graphical Determination of 't'.—Originally the time (t min), from which enzyme activity was calculated, was defined as the period in which the corrected starch iodine colour declined from a value equal to that of the starch substrate-blank to half of that value.[5] The value of the blank colour (E_{1cm}565nm), was normally close to 1·00, and under these conditions the definition is acceptable. However in some instances substrate solutions, prepared normally, gave blank values which departed appreciably from 1·00. These discrepancies were traced to the differing moisture contents of different samples of starch.

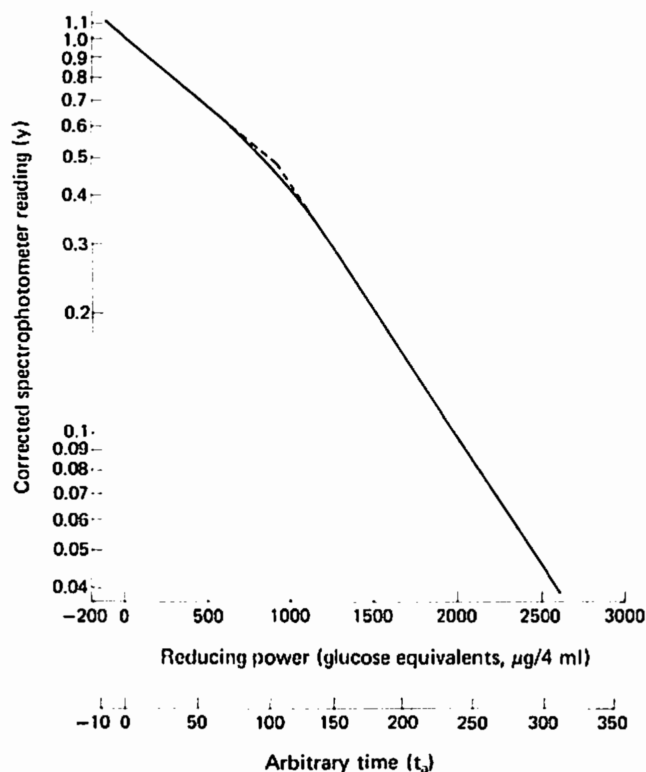

Fig. 1. Corrected spectrophotometer readings plotted on a logarithmic scale against arbitrary time, and reducing power (both on linear scales).

Subsequently the fresh weights of starch used in making substrate solutions were varied slightly so that the substrate blanks in iodine colour values were as close to 1·00 as possible, and at least within the range 0·98–1·02.

A more worrying anomaly, occasionally encountered, was when the straight line drawn through the plot of arbitrary colour values plotted against experimental time failed to intersect the arbitrary colour scale, at zero time, at exactly the value of the substrate blank. The major cause of this problem appeared to be the impossibility of obtaining instantaneous mixing between the enzyme and substrate solutions, giving incorrect "zero times" for the start of the reactions. Vigorous mixing at the inception of each digestion has minimised this problem. The residual problems associated with this and slight real differences between substrate blanks have been overcome by an improved method of calculation.

As before,[5] arbitrary colour values (calculated from the starch-iodine colours using the standard graph) are plotted against experimental time. When the straight line fails to intersect the starch blank at zero time by a large amount the calculations are checked and if these appear to be sound, then the assay may be repeated. However if the discrepancy is small the line defined by the samples from the digestion mixture is accepted. Now the value of t is redefined as the time (min) in which the corrected starch-iodine colour values (E_{1cm}565nm) decline from 1·000 to 0·500 (equivalent to 0 and 4·29 respectively on our arbitrary colour scale). This period, t, is determined graphically (Fig. 2) and enzyme activity is calculated as 100/t S.I.C. units, as before. While this method of calculation is preferable to that originally described it still involves the use of the standard graph, which is laborious.

By experiment, 1 S.I.C. unit of α-amylase causes an increase in reducing power equivalent to 484 μg glucose/h/4 ml reaction mixture (mean of two concordant sets of determinations, separated by 10 years). The initial rate of increase in reducing power is linear for a substantial period of time. Thus a simple factor may be used to interconvert S.I.C. units and reducing power units. The period t used in calculating enzyme activities in S.I.C. units is equivalent to 96·5 arbitrary time units, since from our standard graph 96·5 arbitrary time units elapse while the corrected spectrophotometric readings (E_{1cm}565nm), of the starch iodine colours decline from 1·000 to 0·500 (Fig. 3). One S.I.C. unit of α-amylase causes an increase in reducing power of 484 μg glucose equivalents/h/4 ml reaction mixture. For 1 S.I.C. unit of enzyme activity t = 100 min (96·5 arbitrary

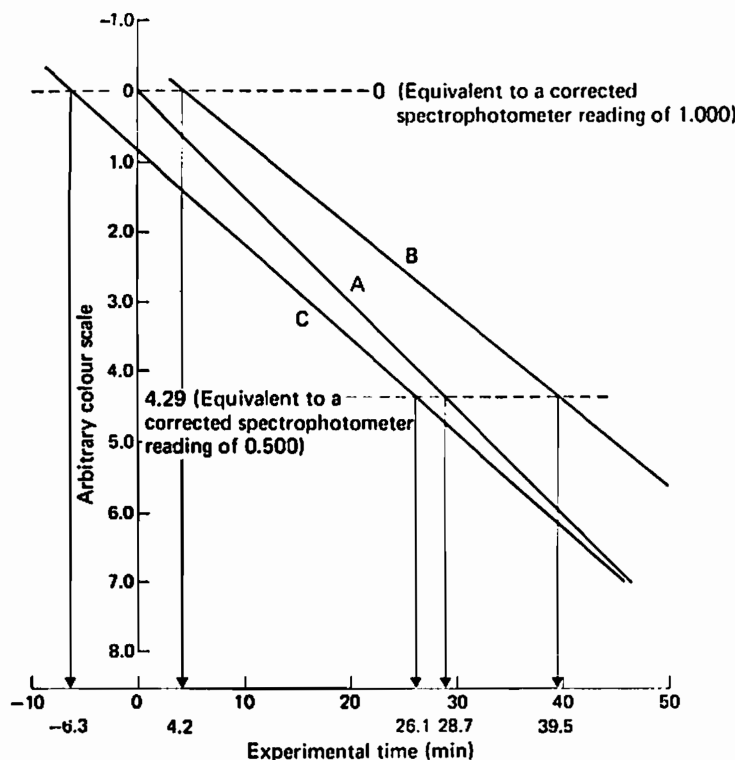

Fig. 2. A diagram illustrating the revised method of finding t from graphs of arbitrary colour values against time. For these graphs the values of t are, A = 28·7 min; B (39·5 − 4·2) = 35·3 min; C (26·1 + 6·3) = 32·4 min.

time units). During this time the reducing power of the digest increases by 484 × 100/60 = 806·67 μg glucose equivalents/4 ml. The factor of 100/60 is involved because the rate of increase in reducing power is per 60 min. Thus the passage of 1 arbitrary time unit coincides with an increase in reducing power of 806·67/96·5 = 8·36 μg glucose equivalents/4 ml. Thus arbitrary time (t_a) or the initial rate of increasing reducing power can be plotted against corrected spectrophotometric readings (y) of starch-iodine colour (Fig. 1, 3).

As alternatives one may find (a) reducing power units and (b) arbitrary times equivalent to particular corrected spectrophotometer readings from the graph (Fig. 3), plot them against sample times, and find either (a) the activity (rate of increase in reducing power) directly from the slope of the straight line, or (b) t, the time in min. for the passage of 96·5 arbitrary time units, from which activity in S.I.C. units can be calculated.

The Use of a Programmable Electronic Calculator in Computing Enzyme Activities.—An alternative method of calculation is based on an equation directly relating corrected spectrophotometric readings with arbitrary time values (or the initial rate of increase in reducing power in enzyme digests), and the use of a calculator to solve the equation for each experimental result. The following empirical equation was derived, which relates t_a to the corrected starch-iodine colour, y:

$$t_a = \frac{-0{\cdot}1229}{y^2} + \frac{8{\cdot}096}{y} + 191{\cdot}76 - 244{\cdot}99y + 45{\cdot}88y^2$$

Multiplication of t_a by 8·36 gives equivalent values in reducing power (glucose equivalents, μg/4 ml). The coefficients in this equation were chosen to give an exact fit to the experimentally determined standard curve at five suitably spaced points. The form of the equation, with terms in y^{-2} and y^{-1}, was dictated by the need to accommodate the very steep increase in t_a as y falls below 0·1. A fourth order polynomial, which might seem a more obvious alternative, proved unsatisfactory because it could not give an acceptable fit in this region.

A Compucorp 445 Statistician electronic calculator was programmed so that, when supplied with readings for enzyme and substrate blanks, and uncorrected spectrophotometer readings of sample-iodine colours it corrected the raw data for the blanks and solved the equation to deliver either the equivalent arbitrary time (t_a) or reducing power (glucose equivalents, μg/4 ml) values. The agreement between results obtained from the calculator and from the standard graph is excellent, even at the early stages of a reaction (Fig. 4). Now experimental sample times are plotted manually against "arbitrary times" obtained from the calculator. From the graph the time interval (t min) is found in which the arbitrary time changes by 96·5 units, and the result is calculated as 100/t S.I.C. units. Alternatively, if preferred, reducing power values are plotted against time, and enzyme activity is expressed as the initial increase in reducing power per unit time. The method of plotting the results manually is still used so that a visual check on the "goodness of fit" of the results to a straight line is obtained.

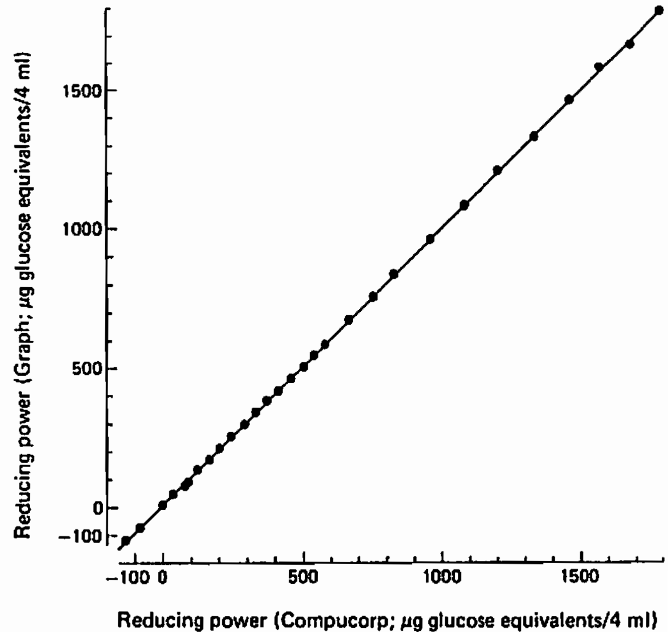

Fig. 4. Reducing powers equivalent to various corrected spectrophotometric readings, found using the standard graph,[5] plotted against the equivalent values calculated using the empirical equation and the programmed Compucorp Statistician calculator. The straight line is the theoretical relationship between these values.

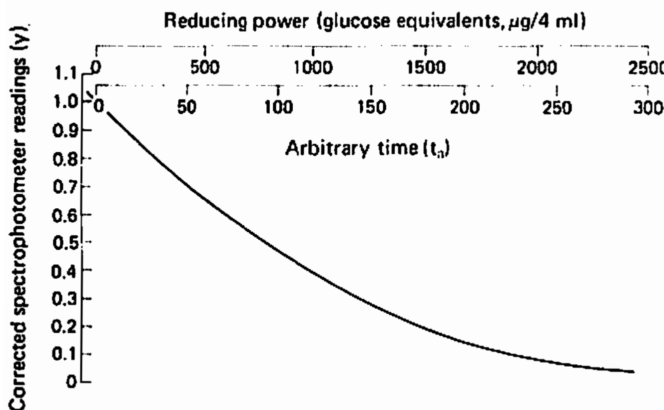

Fig. 3. Corrected spectrophotometer readings plotted on a linear scale against arbitrary time, and reducing power (also on linear scales).

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