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Technical note

Reliability factor for identification of amyolytic enzyme activity in the optimized starch-iodine assay



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ABSTRACT

Amyolytic enzymes are a group of proteins degrading starch to its constitutional units. For high-throughput screening, simple yet accurate methods in addition to the reducing ends assays are required. In this article, the iodine assay, a photometric assay based on the intensely colored starch-iodine complex, was adapted to enable accurate and objective differentiation between enzyme and background activity using a newly introduced mathematical factor. The method was further improved by designing a simple setup for multiple time point detection and discussing the applicability of single wavelength measurements.

One of the most important sources of carbohydrates for human diet is starch [1], a structure entirely built up of glucose units interlinked via α -1,4- and α -1,6-glucosidic bonds, forming linear chains and branch points, respectively. This polysaccharide consists of the linear amylose and branched amylopectin in a highly varying ratio ranging from below 15% to about 40% amylose [2]. Starch is biochemically degraded by amyolytic enzymes such as α - and β -amylases as well as amyloglucosidases [3].

Typically, quantitative determination of catalytic activity of amyolytic enzymes is conducted via estimation of the amount of formed reducing sugars (e.g. glucose or maltose) by using methods such as the Nelson-Somogyi [4] and dinitrosalicylic acid (DNS) assay [5]. Another frequently used method is the iodine assay [6,7]. This photometric method is often applied for quick determination of the activity of starch converting enzymes [6] and is based on the intensely colored complex between iodine and linear chains in starch long enough to form helices [8]. The wavelength of absorbance maximum (λ_{\max}) of the starch-iodine complex's color is governed by the chain length (DP) of the oligosaccharide and shifts from about 490 nm (DP 18) to around 600 nm (DP 72) [9]. This behavior not only results in a high diversity of λ_{\max} between different starches [10] but also causes a shift in λ_{\max} in response to the activity of amyolytic enzymes [11], causing a considerable error in estimated activity when not addressed. Although the iodine assay has been developed decades ago [6,11], optimizations are scarce and mostly designed for single-point measurements [7]. Due to its speed and specificity for long glucan chains, however, the iodine assay has great potential for being a screening method. The aim of this

study was to design a method for the measurement of multiple time points that is fast (< 30 min), easy (stable stock solutions) and accurate. Further, challenges for the interpretation of the data regarding the shift in λ_{\max} caused by some amyolytic enzymes and the differentiation between background and enzyme activity were both addressed by proposing a procedure and mathematical formula, respectively.

Firstly, the method was optimized regarding factors such sample volume, substrate concentration, and the aliquot volume. The developed method was conducted on two microtiter plates and using one row or column/sample. Appropriate amounts of enzyme was diluted to 150 μ l with 50 mM sodium phosphate buffer, pH 6.0 in a microtiter plate and incubated in a water bath at 40 °C for optimal heat transfer. The enzyme reaction was started by adding 50 μ l substrate solution (4 mg/ml, diluted with buffer) to the enzyme solution. Then, at every full min, 15 μ l aliquots of the enzyme reactions were transferred to the analysis wells located on a plate at RT containing 100 μ l freshly prepared iodine reagent (0.15% KI, 0.015% I₂ from stock (26% KI, 2.6% I₂ [12]) with 5 mM HCl), followed by a brief washing steps for the pipette tips using the washing wells (200 μ l buffer). In between transfer, the plate containing the analysis wells was covered with a plastic lid to prevent color depletion. After transfer of the last aliquot, the absorbance of the samples in the analysis wells was detected at 610 nm and a spectrum was measured from 450 to 750 nm with 5 nm steps using a spectrophotometer (SpectraMax from Molecular Devices) and the enzyme activity was calculated as 1 U = -1 ABS/min. This activity unit was found suitable for quick determination of activity. For more accurate values, it is recommended to estimate the corresponding

Abbreviations: DMSO, dimethyl sulphoxide; λ_{\max} , absorbance maximum; F_{Rel} , Reliability Factor

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substrate concentration using a standard curve (Figure S1) [7].

Starches are typically challenging to dissolve in aqueous solutions and tend to have a rather limited stability [13]. Starch solutions in dimethyl sulphoxide (DMSO) on the other hand can be used as stock solutions as they are stable for several months [14], not only simplifying the method but also increasing the comparability between separate experiments. Starches were either found to be soluble at 100 mg/ml in pure DMSO (high amylose content) or at 25 mg/ml in 90% DMSO (low amylose content) and were thus concentrated enough to prevent inhibition of enzyme activity by DMSO [13–15].

Regarding the optimal wavelength for measurement, four starch substrates (potato amylose, Hylon VII, potato and rice starch) were tested. The estimated λ_{\max} of their complex with iodine ranged from potato amylose ($\lambda_{\max} = 641 \pm 8$ nm), over Hylon VII ($\lambda_{\max} = 590 \pm 10$ nm) and potato starch ($\lambda_{\max} = 588 \pm 12$ nm) to rice starch ($\lambda_{\max} = 562 \pm 16$ nm), being in good agreement with previous data [10]. Therefore, the wavelength of 610 nm was selected as it enabled detection of all substrates with a single wavelength (< 3% difference in the range 580–640 nm). The activity of amyolytic enzymes on starch was found to cause a blue-shift in λ_{\max} of varying strength, ranging from β -amylases (~25 nm/ABS on potato amylose, Fig. 1B) to α -amylases (~100 nm/ABS, Fig. 1A, for the full list see Figure S2 and S3). Fig. 1C shows the underestimation of the absolute absorbance compared to the relative absorbance measured at 610 nm of all ten studied amyolytic enzymes on potato amylose. Above a λ_{\max} of about 570 nm, the difference between the absolute and relative absorbance values was minimal (below 10%). The underestimation of absorbance led to an overestimation of activity due to a predicted faster decrease in absorbance. If the λ_{\max} fell below 570 nm, the activity overestimation was up to 25% which was observed for e.g. α -amylase

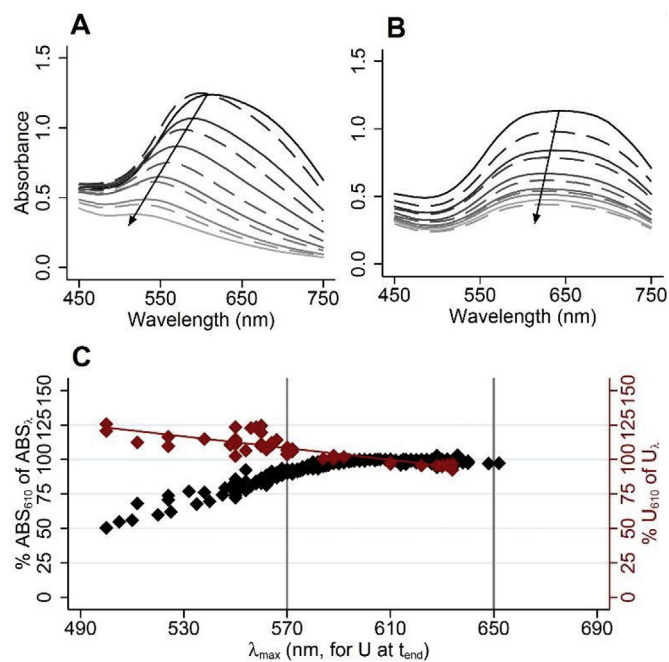


Fig. 1. Detected shifts in absorbance maximum (λ_{\max}) in response to enzyme activity on amylose. Absorbance over time during incubation with (A) α -amylase (*A. oryzae*) and (B) β -amylase (*B. cereus*) showing one spectrum/min as mean ($n = 3$) and λ_{\max} is indicated by arrows. (C) Correlation between λ_{\max} , absorbance values measured at 610 nm and derived enzyme activity (U) of ten amyolytic enzymes. Y-axes show percentages of values at 610 nm compared to the values at λ_{\max} . For absorbance values, each time point is indicated in black, while estimated enzyme activities (red) represent entire time series. Gray lines frame the recommended range for single-wavelength measurements. For conditions see Supplementary Data. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

on amylose below approx. 0.6 ABS at 610 nm. In this case it was recommended to estimate the enzyme activity based on the absorbance values at the λ_{\max} of each time point. For all experiments in which all data points exhibited a λ_{\max} within the given range, however, the overestimation was below 10%, providing a sufficiently narrow window for accurate detection of enzyme activity at a single wavelength.

The assay was designed for detection of the enzyme reaction at multiple time points as the enzyme activity was not always found to be identical throughout the entire assay time frame, rendering single-point analysis inaccurate, especially for low enzyme activity or non-linear trends (deceleration). Those two types of reactions are often difficult to differentiate from background activity. Therefore, investigations were conducted to establish a factor that could reliably distinguish between enzyme and background activity. Since the method was aimed for an array of amyolytic enzymes on a series of starches, the factor should be applicable to a range of initial absorbance values, length of incubation, and enzyme type. Analysis of a large dataset of ten amyolytic enzymes and negative controls on amylose ($n = 97$, see Supplementary Data) revealed that the main differences between background and enzyme activity were the linearity and level of decrease in absorbance. The linearity of the decrease was determined by the coefficient of determination (R^2) as it shows the percentage of data points of an experiment described by the estimated linear slope [16]. The level of decrease was calculated by using the ratio of the first and last absorbance value (ABS_{Start} and ABS_{End} , respectively) as it was less distorted by different starting absorbance values and non-linear decreases.

In general, high enzyme activity was found to be indicated by a sharp, highly linear decrease in absorbance over time (< -0.075 ABS/min, $R^2 > 0.95$) that could conclude in a deceleration of absorbance decrease due to substrate depletion and thus a lower R^2 value. Background activity, on the other hand, resulted in a series of absorbance values that exhibited small, random fluctuations (about 7.5% of initial absorbance value), almost no decrease over time (> -0.007 ABS/min) and a low linearity ($R^2 < 0.4$). Low enzyme activity generally showed a very linear decrease, however, the fluctuations in absorbance were more visible than for higher enzyme activities. Detection of low enzyme activity was further impeded by the fact that sometimes the deviations in absorbance of samples with no enzyme activity may seem non-random, giving the appearance of a trend and thus enzyme activity.

Taken together, basing the modeled Reliability Factor (F_{Rel}) on both linearity and level of decrease in absorbance enabled detection of both high and low enzyme activity. Additionally, cutoff values were introduced based on the careful manual identification on presence or absence of enzyme activity of each sample in the dataset (Figure S4). The two components contributing to the F_{Rel} were further weighted similarly to ensure a positive result whenever one of the factors was slightly negative but the other was sufficiently positive (see Equation (1)).

$$F_{Rel} = (ABS_{Start}/1.5 - ABS_{End}) * 1.5 + (R^2 - 0.75) \quad (1)$$

It should be noted that the F_{Rel} provided only information about the reliability of presence or absence of enzyme activity and not the level of activity itself. The F_{Rel} can, however, be used to estimate reliably if the derived activity rates can be trusted.

After establishment of the equation, the model was tested on a control experiment with three amyolytic enzymes as positive controls and two negative controls on four different starchy substrates. Fig. 2A shows the obtained raw data while Fig. 2B presents the estimated F_{Rel} values. Both raw data and F_{Rel} values showed a clear distinction between the positive and negative controls on all substrates. Rice starch exhibited the lowest absorbance values and decreases and thus the smallest F_{Rel} values. β -Amylase showed only very low decreases and F_{Rel} values with two of them even being slightly negative. This result, however, was not surprising as almost no decrease was visible in the

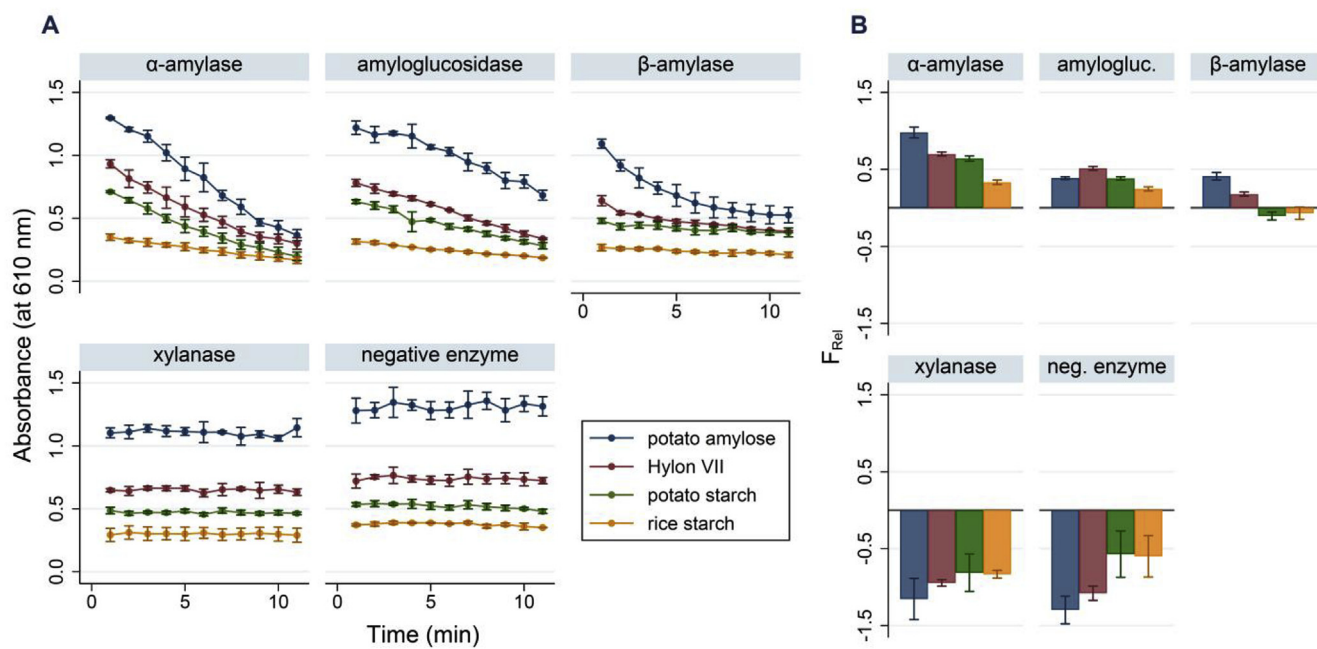


Fig. 2. Observed activities of control experiments with (A) absorbance values over time and (B) estimated Reliability Factors (F_{Rel}). There were three positive controls (0.2 $\mu\text{g/ml}$ α -amylase from *A. oryzae*, 6 $\mu\text{g/ml}$ amyloglucosidase from *A. niger* and 4 $\mu\text{g/ml}$ β -amylase from *B. cereus*; all purchased from Megazyme) and two negative controls (63 $\mu\text{g/ml}$ xylanase from *A. niger* (Megazyme) and negative enzyme) incubated with 1 mg/ml substrate (potato amylose, potato and rice starch from Sigma-Aldrich and Hylon[®] VII from Ingredion). Data are presented as mean \pm stdev ($n = 3$).

raw data. Further, the observed non-linear trend for β -amylase indicating early substrate depletion was in agreement with its inability to bypass or cleave α -1,6-linkages [11,17]. Notably, statistical results (Pearson correlation, Table S1) estimated a significant correlation (linear regression) between time and absorbance values for all positive controls but none of the negative controls at a confidence interval (CI) of 99.9%, suggesting presence of enzyme activity even for β -amylase on rice starch. However, there was also a significant correlation between negative enzyme and potato starch at a CI of 99.5% ($p = 0.002$). Therefore, the parameters for the F_{Rel} were not altered accordingly to prevent false positives. Additionally, F_{Rel} values very close to zero indicate the requirement for a repetition of the experiment to obtain a more defined result.

In summary, a new procedure for the iodine assay has been proposed to conduct fast and easy experiments with multiple time points. All data points could be measured at a single wavelength (610 nm) whenever their absorbance maxima were within the range of 570–650 nm, providing accurate activity profiles of an array of amylolytic enzymes. Furthermore, a novel variable, the Reliability Factor, has been designed to provide an objective and simple way to distinguish between enzyme activity and background activity and its application was verified on both positive and negative controls on four starchy substrates.

Author statement

Aline L. O. Gaenssle: Data curation, Investigation, Formal analysis, Methodology, Writing – Original Draft, Validation, Visualization

Marc J. E. C. van der Maarel: Conceptualization, Funding acquisition, Resources, Project administration, Supervision

Edita Jurak: Conceptualization, Project administration, Supervision, Writing – Review & Editing

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ab.2020.113696>.

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