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Viscometric Measurement of Protease Activities on Gelatine Substrate

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Abstract:
The knowledge of enzymatic activity is necessary in many industrial processes. The common measurement techniques are time-consuming and therefore cost-intensive. Measurements of viscosities are a promising approach as a fast and cheap testing method. The major challenges are to find a suitable substrate with Newtonian flow behavior throughout the whole testing range as well as a correlation between viscosity of the solution and the decomposed mass. Water based gelatine-buffer-system as substrate is tested extensively regarding the dependence on different solvents, pH-values and gelatine batches. All viscosity measurements are performed with a rotational viscometer. It is shown that the gelatine-buffer-system is independent of the given parameters and found to fulfill the said requirements. A correlation model based on the Martin equation and necessary assumptions are presented. The required parameters intrinsic viscosity and Martin parameter can be derived by few measurements with little effort. The digesting enzyme Trypsin is used as model enzyme in the degradation experiments. The enzyme concentration is varied and the decrease of the viscosity is measured. A dependency between the enzyme concentration and the enzymatic activity or respectively the viscosity decrease is observed.

Key words:
Enzyme activity measurement, protease, trypsin, gelatine, intrinsic viscosity, rotational viscometer, Martin equation, correlation model, degradation of gelatine solution

1 INTRODUCTION

Many products used in everyday life are produced with the aid of enzymes, e.g. food and beverages, detergents, textiles and recycled paper. Enzymes are biological catalysts for accelerating chemical reactions under mild conditions and are favored for their high selectivity. Therefore determining the catalytic activity of enzymes is a matter of importance. The absolute activity of enzymes is defined as the amount of substrate converted during the catalyzed reaction per time (reaction rate) in the SI-unit katal (mol/s). The specific activity is the degradation rate on a standard substrate, here gelatine, given in digesting units (1 U/mg = 1 μmol/min mg). Another quantity to describe the enzyme activity is the apparent reaction rate in 1/s. This is most common during the development of a viscometric activity measurement method. For each enzyme a maximum efficiency appears at an optimum pH-value as well as at an optimum temperature. Hence enzyme activities are always related to the present conditions during the measurement as well as the used substrate.

Quantification of enzymatic activity is mostly based on spectrophotometric and calorimetric methods, therefore general enzyme activity measurements are cost intensive and time-consuming. As an example the determination of collagenase activity is carried out by observing the transient concentration shift when converting an artificial protein by measuring the change of photometric absorption of the sample. The reaction of the samples taken has to be stopped by adding citric acid. In the next step an extraction is performed with ethyl-acetate. The absorption of the organic phase is measured and related to the substrate concentration with a predetermined calibrating curve [1]. Affordable rapid testing methods would be very beneficial, e.g. for production processes in the detergent industry. Fast results during process operation could facilitate accurately meeting the product specifications. Viscometric methods to measure enzymatic activity provide a promising approach to this problem due to ordinary relations between substrate concentration and solution viscosity. Different methods [2–6] were developed to determine enzymatic activity

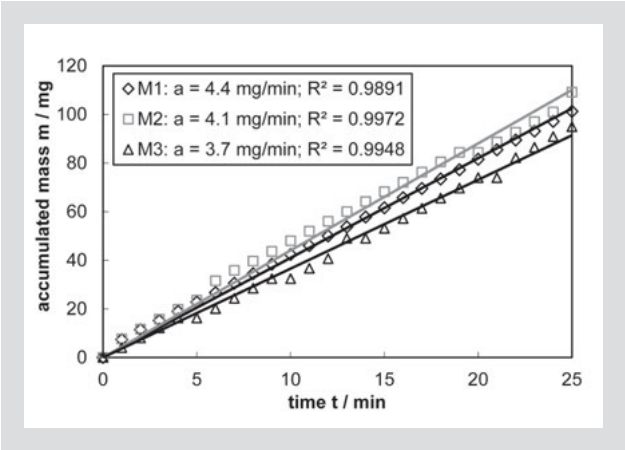

Figure 8: Degraded substrate m depending on time.

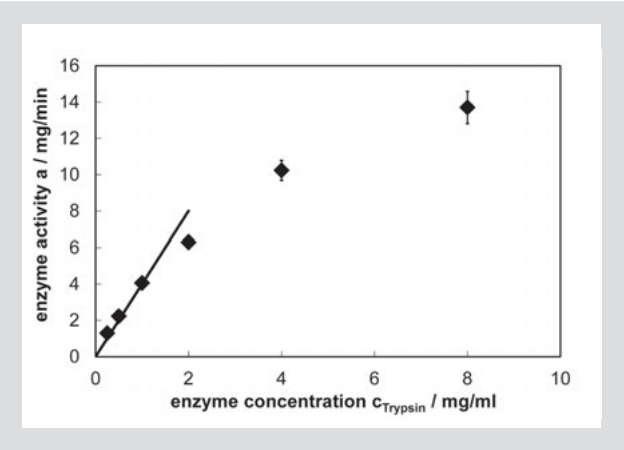

Figure 9: Dependence of enzyme activity at different enzyme concentrations $c_{Trypsin}$.

gelatine batch 2003001 dissolved in buffer 2 with addition of NaOH was used for the degradation experiments. The intrinsic viscosity for this system is $[\eta] \approx 5.36$ ml/g and the Martin parameter amounts $k_M \approx 0.29$ as given in Table 5.

4.2 DEGRADATION EXPERIMENTS

The influence of shear as well as addition of buffer and of an inactive enzyme to the viscosity of the gelatine buffer solution show constant values after 7–10 minutes. Even though the viscosity had dropped before due to dilution. The decrease of the viscosity during the degradation experiments is solely attributed to the enzyme activity. Figure 7 shows the temporal dependence of the gelatine-buffer-solution viscosity after addition of the enzyme with a concentration of $c_{Trypsin}$ = 1 mg/ml. The three curves are very similar, only the initial viscosity and the increase of viscosity at the beginning are conspicuous. These deviations are linked to the evaporation during the storage time of the gelatine-buffer-solution. However, the differences in the initial viscosity have no influence to the degradation experiments, because only the temporal degradation of substrates is important to determine the enzyme activity.

The results of the decomposed gelatine mass from the above given sample are shown in Figure 8. The linear part of the viscosity-plot in Figure 7 was evaluated. The degraded substrate mass was calculated according to Equation 5 and accumulated over time. The resulting curves were shifted to zero time for better comparison. The activities extracted from the slope of the curve are between values of 3.7 and 4.4 mg/min. The coefficients of determination are at least 99 % and demonstrate acceptable accuracy. The viscosity measurements for the other concentrations $c_{Trypsin}$ = 0.25, 0.5, 2, 4, and 8 mg/ml provide reproducible results as well. Decomposed amounts and measured activities in terms of their mean values and standard deviations are given in Table 6. The Michaelis-Menten-kinetics [14] assumes that in the range of low enzyme concentrations the enzyme activity doubles if the enzyme concentration is duplicated. In Table 6 the activity is doubled up to a concentration of $c_{Trypsin}$ = 1 mg/ml by duplicating the amount of enzyme. Beyond that this assumption is not confirmed. Increasing deviations for smaller concentrations can be observed. This could indicate an insufficient sensitivity for small enzyme concentrations. Further measurements are needed to determinate the sensitivity of this measurement method with other, especially lower enzyme concentrations and other enzymes.

$c_{Trypsin}$	(mg/ml)	0.25	0.5	1	2	4	8
average activity *a*	mg/min	1.3	2.23	4.07	6.30	10.23	13.7
abs. deviation Δa	mg/min	0.26	0.12	0.31	0.1	0.55	0.89
rel. deviation Δa	%	20.0	5.38	7.69	1.59	5.38	6.5

Table 6: Concentrations of enzymes and the determined activities.

Figure 9 enables a closer consideration of the dependence of the viscosity decrease on the enzyme concentration. In this graph the average activities are shown as a function of concentration. The dependence of enzyme activity in the range of lower concentrations is linear, but for larger concentrations the activity change is still sensitive but less. This limit describes the point in which all binding sites at the substrate are involved and the solution is saturated with enzymes. A further increase in the amount of enzyme has no further influence on the activity.

5 CONCLUSIONS

The results of this study have shown the applicability of a viscometric method for determining enzymatic activity related to decomposed mass of gelatine. Highly soluble non-gelling gelatine powder was used as substrate and dissolved in 50 mM phosphate buffered saline solution. The endo-protease Trypsine was used as model enzyme. Sodium hydroxide (NaOH) was added to the solution in order to adjust a pH-value of 7.4, to match the optimal activity range of trypsin. Measurements

were conducted in a rotary viscometer at constant shear rate and 20 °C. Enzyme concentrations were adjusted to 0.25, 0.5, 1, 2, 4, and 8 mg/ml at substrate concentration of 1.2 g/ml with a mixing ratio of substrate solution to enzyme solution of 19:1. An important feature of this method is the utilization of a correlation model in order to relate the measured viscosity data during enzymatic degradation experiments to the degraded substrate mass on basis of the Martin equation. Thus, additional measurement systems in order to identify material specific parameters are not needed. Although assumptions were necessary the validity of this model has been displayed partly in theory as well as by clarifying the influence of enzyme concentration on the enzyme activity. It has been demonstrated that the necessary specific parameters, i.e. intrinsic viscosity $[\eta]$ and Martin parameter k_M for the polymer-solvent system could be determined easily by few experiments with the rotational viscometer.

The effect of different buffer compositions in water as solvent, pH-values and gelatine batches on the solution viscosity was investigated. The measurements showed insignificant influences on the rheological properties and therefore on the intrinsic viscosity $[\eta]$ and on the Martin parameter k_M. This demonstrates the stability of the polymer solvent system and the negligible polymeric effect of the used gelatine. In addition, this method may be adapted to other endo-protease enzymes like chymotrypsin and collagenase due to the high variety and quantity of amino acid building blocks in the gelatine. Some important aspects have to be considered when using this method. A minimum viscosity level has to be present during the degradation measurements in order to obtain meaningful data for the evaluation procedure due to the exponential character of the correlation. In addition the amount of cleaved substrate molecules should not exceed a certain amount not to affect the solution viscosity noticeably as this effect is not included in the model.

Further on, cleaving of low molecular weight substrate molecules cannot be detected by this method, but on basis of collision probabilities it is assumed that this amount is negligible. It was observed that relative deviations increase for smaller enzyme concentration. Further investigation is needed in this area in order to identify the sensitivity of this method at low enzyme concentrations. As conditions at constant 20 °C were considered here, the influence of temperature should also be investigated regarding its influence on the degradation kinetics of the enzyme in order to make further progress for realizing an in-process application of enzyme activity determination.

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