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A Method for the Assay of Hydrolytic Enzymes Using Dynamic Light Scattering

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ABSTRACT

Dynamic light-scattering techniques have been successfully used for the assay of several hydrolytic enzymes. The enzymes were assayed using substrate-coated colloidal particles. Hydrolysis of the substrate coat causes destabilization of the particles followed by particle aggregation. The rate of particle aggregation can be related to the initial concentration of added enzyme.

Index Entries: Enzyme; trypsin; papain; α-amylase; amyloglucosidase; DNAase I; dynamic light scattering; assay; analysis; colloidal particles; aggregation; destabilization.

INTRODUCTION

Light-scattering techniques have been used extensively for the measurement of antigen-antibody interactions. Prior to this report, light scattering had not been used for the analysis of hydrolytic enzymes. We wish to report the first application of this technique for the determination of enzyme activities. The assay uses substrate-coated colloidal particles, which when coated remain quite stable for several weeks to months.

The general methodology takes advantage of the fact that unstabilized particles tend to aggregate. Hydrolysis of the substrate coat on the particles causes the particles to become unstable and therefore aggregate. The aggregation rate is proportional to the enzyme concentration.

For these studies we examined two proteases: trypsin and papain; two carbohydrases: amyloglucosidase and α-amylase; and DNAase I.

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The assay protocol was as follows: An enzyme, at an unknown dilution (if a standard curve has been prepared), is introduced into a suspension of the substrate-coated colloidal particles. On introduction of the enzyme, the substrate on the particles begins to hydrolyze, rendering the particles unstable. The unstable uncoated particles begin to aggregate. The rate of aggregation will depend on the concentration of the added enzyme. In the case of nucleic acids (DNA), the particles move from aggregation to disaggregation followed by slow aggregation. The initial disaggregation reaction is the important parameter, which is monitored by light scattering. The aggregation of the particles can be measured by either total scattering or by dynamic scattering. All nephelometric techniques are believed to be usable in this assay, because the total light scattering intensity does increase with time.

DYNAMIC LIGHT SCATTERING FOR HYDROLYTIC ENZYME ASSAYS

Dynamic light-scattering methodology measures a photon autocorrelation function that is initially characterized by a single decay constant whose value is related to the single particle diffusion coefficient. As soon as the particles lose a portion of their substrate coat, they start to aggregate, and the substrate autocorrelation function shows an additional component that has a long time-decay constant. The amount of the component with the long decay (slow) relates to the amount of enzyme introduced into the solution. In the case of the enzymes tested, the approximate limit of sensitivity appears to be in the microgram range.

Preparation of the coated particles (Immunocon Corp., Huntington Valley, PA) was accomplished by adding the substrate solution at approx 0.2% (v/v) to a suspension of colloidal particles with an average size range of 60–100 nm. The mixture was sonicated for 3 min or until the particles were stabilized. The particles in this condition do not aggregate or separate out of solution over several weeks of storage at 4°C.

Coating particles with DNA was carried out in a slightly different fashion. The particles were first sonicated for 3 min, after which the DNA solution was quickly added and mixed with the particles before aggregation could begin. Sonication of the DNA particle mixture caused rupture of the DNA polymeric chains and considerably decreased the stability of the particles.

RESULTS

The results of these experiments show that colloidal particles coated with bovine serum albumin (BSA), starch, or DNA make excellent substrates for the analysis of the hydrolytic enzymes tested. The result of a typical dynamic-light scattering experiment is shown in Fig. 1 for a typical experiment. For this experiment, 25 μg of papain was added to 0.2

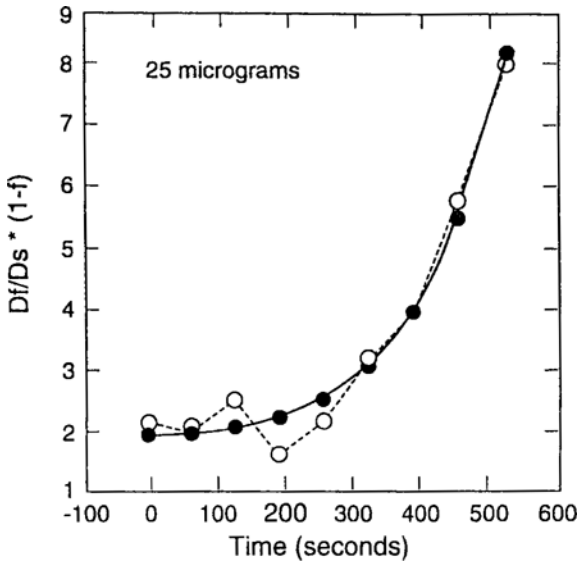

Fig. 1. Increasing particle size plotted as the diffusion coefficient of the slow fraction/diffusion coefficient of the fast fraction × 1 minus the fraction of the fast fraction for a 25-μg addition of papain to 3.0 mL of a colloidal suspension of BSA vs time.

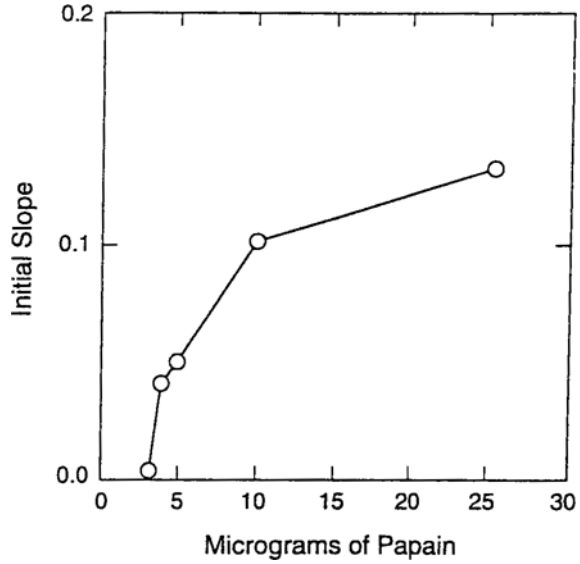

Fig. 2. The magnitude of the initial slopes calculated from data similar to that shown in Fig. 1 for different papain concentrations vs the concentration of papain.

mg/mL of BSA-coated particles. The ratio of the fast/slow diffusion coefficients times the slow fraction (representing increasing particle size) was plotted vs time. The results show an exponential increase in the fraction of large particles with assay time. The magnitude of the exponential for several increasing concentrations of papain is presented in Fig. 2. The

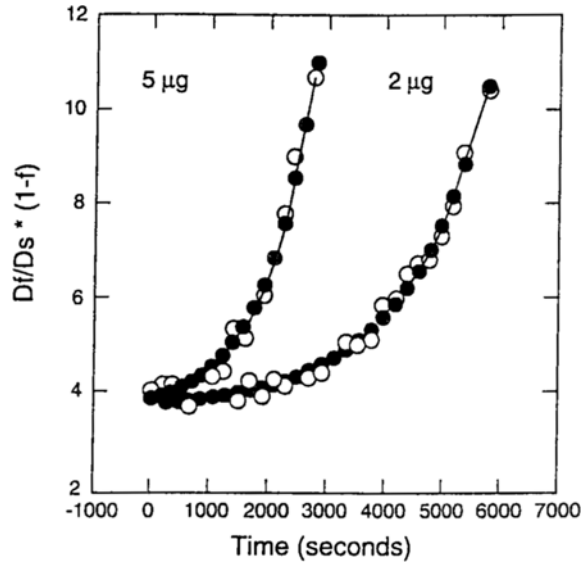

Fig. 3. Plotted as in Fig. 1 for trypsin at two different enzyme concentrations vs time.

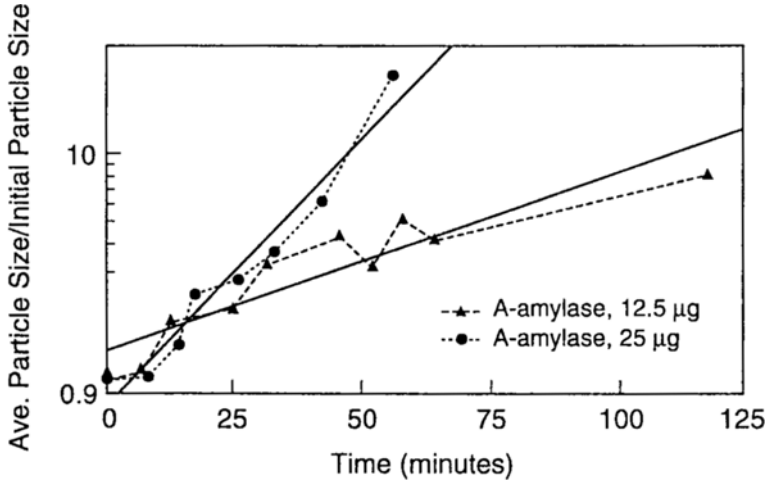

Fig. 4. The average particle diameter change vs time for two concentrations of α-amylase using a suspension of starch bound to colloidal particles.

reaction rate is proportional to the slope, thus showing the enzyme concentration can be determined against a standard curve or on the basis of units of activity/unit time.

In order to demonstrate that the results observed with papain were typical of proteases, we next carried out a series of experiments using trypsin. Typical results are presented in Fig. 3 for two different concentrations of trypsin: 2 and 5 µg added to 3.0 mL of the diluted colloid-coated BSA substrate. The results indicate that reaction rates are proportional to

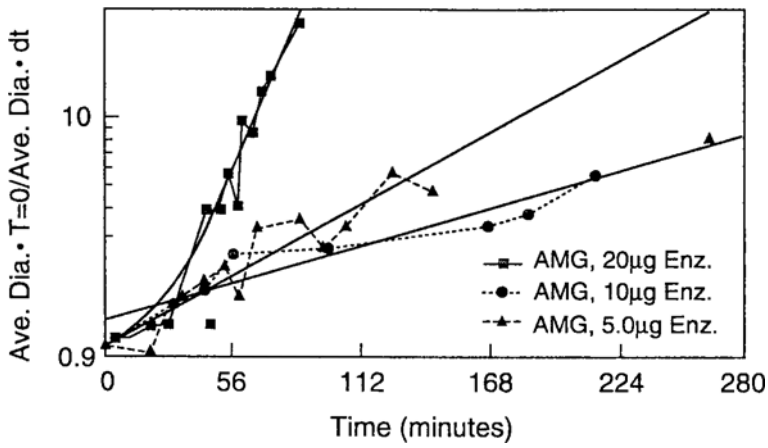

Fig. 5. The average particle diameter change vs time for three concentrations of amyloglucosidase using a suspension of starch bound to colloidal particles.

the enzyme concentration and, as in the case of papain, show an increase in the aggregated particles vs time.

In order to show the versatility of the method, we examined the hydrolysis of starch using two amylases. In these studies, the starch was coated onto the particles at pH 5.5 in 0.2 *M* acetate buffer at a concentration of 0.002 mg/mL. Known concentrations of α-amylase were added to the mixture of starch and colloidal particles. The change in particle size was monitored vs time. The results of these experiments are presented in Fig. 4. The change in particle size, in this case plotted as average particle diameter vs time, shows, as in the case of the proteases, that the reaction rate is proportional to the enzyme concentration of α-amylase.

A similar experiment was carried out using amyloglucosidase. The substrate was prepared as described for the α-amylase experiments. The results, presented in Fig. 5, plotted as average particle size vs time shows that the rate of increase in the particle size is proportional to the concentration of the amyloglucosidase at three different enzyme concentrations.

It is obvious from the data that increasing particle size can also be monitored by total light scattering and by nephelometry. Therefore, this quantitative technique should be useful not only for light scattering, as used here, but also for standard nephelometry.

The assay of DNAase I was carried out in a similar fashion to the other enzymes. However, rather than observing an increase in particle size vs time, we observed a decrease in particle size vs time. The experiments were carried out in 0.2 *M* acetate buffer, pH 4.5, containing 5 m*M* $MgSO_4$. The results for three DNAase I concentrations are presented in Fig. 6. The data show that the rate of decrease in particle size is proportional to enzyme concentration. In this case, unlike the cases of the other enzymes, the particles are initially aggregated by binding to the long

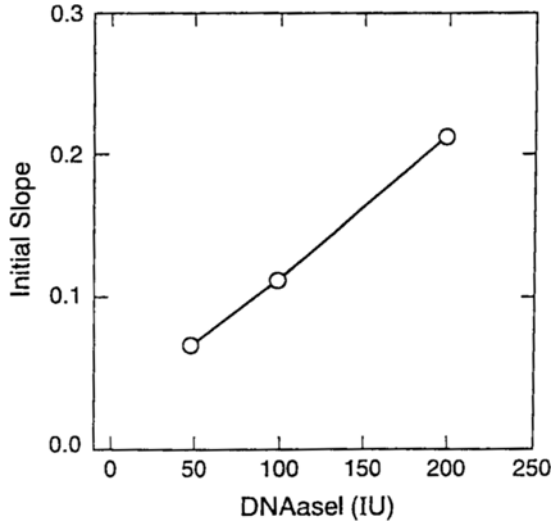

Fig. 6. The magnitude of the initial slopes of the disaggregation rate for DNAase I vs different concentrations of the enzyme.

DNA molecules that hold the particles together. The aggregation can be eliminated by sonication. However, the sonicated particles do not appear to work well as an aggregating substrate for the enzyme, most likely because the molecules are already fragmented by the sonication process. The disaggregation observed on addition of the DNAase I is likely caused by the initial cleavage of the DNA holding the particles together. These molecules would be the most exposed and, therefore, the most likely to be initially attacked by the enzyme.

SUMMARY

A method has been presented for performing an enzyme assay using dynamic light-scattering techniques. Substrate-coated colloidal particle were diluted in a buffer to a useful concentration range that gave an acceptable signal. The assay was initiated by adding the enzyme to be assayed. Substrate hydrolysis on the coated particles cause a change in the particles that can be monitored using light-scattering techniques. When enzymatic hydrolysis occurs, the stability of the coated particles are disturbed, and either aggregation or disaggregation occurs.