The Use of a Digital Oscillator Densimeter for the Determination of Reaction Rates of Bacterial Proteases

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In this work, we introduce an accurate, simple, and practical method for determining the reaction rate of proteolytic enzymes. The reaction rates of bacterial proteases were measured by analysis of casein hydrolysis using the digital oscillator densimeter technique.

Experimental

1. Materials

Casein (according to Hammersten) was obtained from Merck, West Germany.

Alcalase and Maxatase were thermostable bacterial proteolytic enzyme preparations produced as granules from special strains of Bacillus which are used in detergents at high temperatures and high pH values. These enzyme preparations were kindly provided by Novo Industry A/S, Bagsvaed, Denmark, and by Gist-Brocades N.V. Industry, Delft, Holland.

2. Methods

Determination of the density of the substrate solution. The density of casein solutions (1-5%) were measured in the pH range 6.2-9.2 and over the temperature range 37-60 °C by a digital densimeter (DMA O2C, A. Paar Graz, Austria [7, 8a] using water as a reference, which leads to the most accurate results [8b]. The principle of the technique is based on the determination of the natural frequency of an electronically excited mechanical oscillator filled with the liquid sample. The density of the sample, d was calculated from the equation: $d = A t^2 + B$, where $t$ is the period in seconds of the oscillator when filled with the sample. A and B are constants determined by calibration measurements using air and distilled water [9].

Procedure for the rate of protease-catalyzed reaction measurements. The determination is based on the change in density of the substrate-enzyme mixture measured by the densimeter as above. The principle of the technique is that the hydrolysis of casein by protease consumes water, which causes a decrease in the volume of the casein-protease mixture and subsequent increase in density according to the equation:

\[ \text{Casein + water} \xrightarrow{\text{protease}} \text{amino-acids + peptides}. \]

Casein solution (5%) at pH 8.2 and protease solutions at pH 8.2 were pre-incubated separately at 37 °C. The dried oscillator was filled from a mixture of 5 ml of casein solution and 1 ml of protease solution...
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Fig. 1. Effect of enzyme concentration on density of reaction mixture using Maxatase at pH 8.2 and 37 °C

dilution. The first density measurement was carried out after 8 min, which was necessary for thermal equilibration. The increase in density was measured periodically at intervals of 0.5 min for 30 min. The density was calculated according to the equation previously shown. The dependence of density on the time of the enzymic reaction was plotted by computer (Hewlett Packard Model 9810A) equipped with a plotter for different concentration of protease (Fig 1). Enzyme reaction rate ($\frac{d}{d\text{t}}$) using different protease concentrations were calculated. $d$= the difference between the densities of two subsequent points on the density/time curve (Fig. 1), $\Delta t$= the period of time on the abscissa corresponding to $\Delta t$.

The protease activity was determined by the modified Lohlein-Wolhard method used by Kuntzel [11]. The undigested casein was precipitated with a known volume of standard solution of HCl and sodium sulphate, then filtered off, and the filtrate was neutralised by alkali solution. The volume of the alkali solution required to neutralise the acidity of the filtrate was used as a direct measurement of the enzyme activity. The latter was calculated from the standard graph of Alcalase activity.

Results and Discussion

1. Density Measurement of Different Casein Solutions

Densities of casein solutions between 1 and 5%, w/v at pH 7.0 and 40 °C were measured using distilled water as reference. The results are given in Table 1. No measurements of density of casein solutions are reported in the literature.

2. Density Determination of Casein Solution at Different Temperatures

Densities of 5% casein solution at pH 8.2 and over temperature range from 37 to 60 °C were measured. The results are shown in Table 2.

As seen from Table 2, there is decrease in the density of a casein solution on raising the temperature.

3. Density Determination of Casein Solution at Different pH Values

Densities of 5% casein solutions at 40 °C and various pH values from 6.2 to 9.2 were measured. The results are shown in Table 3.

From Table 3, it is clear that there is a slight increase in the density of a casein solution on increasing the pH.

The results shown in Tables 1, 2, and 3 indicate that this method is reliable, and can be used for density measurements of casein solutions.

4. Determination of the Rate of Protease-Catalysed Reaction

The enzyme reaction rates of the bacterial proteases, Maxatase and Alcalase, were carried out at pH 8.2 and 37 °C. The results are shown in Table 4. Table 4 gave slight differences between the reaction rates of Maxatase and Alcalase.