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Kinetic Assay of Chymotrypsin with an Ammonia Gas-Sensing Electrode

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A simple kinetic method for chymotrypsin assay is described. The method is based upon the hydrolysis of *N*-benzoyl-L-tyrosinamide as a substrate, resulting in the production of ammonia. The initial rate of ammonia evolution is selectively measured potentiometrically with an ammonia gas-sensing electrode (Horiba ammonia gas-sensing electrode 5002A-06T). Optimal conditions for the assay are specified. The initial rate of ammonia evolution is proportional to the enzyme activity, and a working range of 0.08 to 0.32 U/ml chymotrypsin was obtained. The recovery of chymotrypsin was 99.2—102.5%. In order to study the applicability of the method, recovery experiments were performed by use of an ophthalmic chymotrypsin preparation.

Keywords——chymotrypsin; *N*-benzoyl-L-tyrosinamide; ammonia gas-sensing electrode; kinetic method

Chymotrypsin is formed in the pancreas and plays an important role in digestion by assisting the hydrolysis of proteins in the intestinal tract. It has been used for the relief of inflammation as an anti-inflammatory enzyme preparation. Screening tests for chymotrypsin are also useful in the diagnosis of pancreatitis and cancer of the pancreas. Therefore, chymotrypsin assay is of great importance in the medical and pharmaceutical fields. Since chymotrypsin partially hydrolyzes polypeptide chains, a large number of substrates have been used in chymotrypsin assays. For example, casein[1] is hydrolyzed by chymotrypsin to form a hydrolysis product, the tyrosine content of which can be measured spectrophotometrically. Synthetic esters or amides[2] are also hydrolyzed as substrates by chymotrypsin and the hydrolysis products can be assayed by titration[3] or spectrophotometric methods.[4] These methods involve secondary reactions that suffer interference from degradation substrate products. The lack of simple, specific, and interference-free methods has restricted the usefulness of the assays. In recent years, the use of gas-permeable membrane electrodes has rapidly increased in the analytical field. This electrode method does not suffer from the turbidity problems that can be encountered in determining fecal samples and protein degradation products would not affect this procedure because of the separation effect of the gas-permeable membrane. Therefore, in this paper we describe a simple and rapid kinetic method for chymotrypsin assay employing an ammonia gas-sensing electrode. *N*-Benzoyl-L-tyrosinamide[5] was used as the substrate, since its hydrolysis by chymotrypsin produces ammonia. The initial rate of ammonia evolution is selectively measured potentiometrically with an ammonia gas sensor. The initial rate of change in potential, with a constant concentration of *N*-benzoyl-L-tyrosinamide, is directly proportional to the enzyme activity present.

Experimental

Apparatus——The ammonia gas-sensing electrode (Horiba ammonia gas-sensing electrode 5002A-06T) consisted of an ammonia gas-permeable membrane, a glass pH electrode and a silver-silver chloride reference electrode

with 0.1 M ammonium chloride as the internal electrolyte. Potentiometric rate measurements were carried out with a Hitachi-Horiba F-7ss pH/mV meter. The measuring cell (10 ml) consisted of the sensor dipped into a sample solution, which was stirred magnetically. The cell, which could be made gas-tight to prevent the escape of ammonia, was kept at a constant temperature with circulating water thermostated by Sharp TE-104 constant-temperature circulator. Initial rate readings were recorded on a Toa Electronic EPR-221E polyrecorder operated at a chart speed of 20 mm/min and a range of ± 50 mV.

Reagents——Type II α-chymotrypsin (E.C. 3.4.21.1) from bovine pancreas, with an activity of 45 U/mg solid (one unit will hydrolyze 1.0 μmol of *N*-benzoyl-L-tyrosine ethyl ester per min at pH 7.8, 25 °C), *N*-benzoyl-L-tyrosinamide, and *N*-benzoyl-L-tyrosine ethyl ester were obtained from Sigma. Zonolysin (ophthalmic α-chymotrypsin preparation) was obtained from Mochida Seiyaku Co. Other chemical substances used were of extra pure grade. Water was distilled in an all-glass apparatus. A substrate solution of 0.025 M was prepared by dissolving 71.08 mg of *N*-benzoyl-L-tyrosinamide in 10 ml of 50% (v/v) ethanol. Enzyme measurements were made from aliquots of a stock solution of 10 mg of α-chymotrypsin dissolved in 10 ml of water. Working solutions of substrate or enzyme were prepared using appropriate dilutions with solvent, just before measurements. Tris–HCl buffer (0.2 M, pH 9.25, 0.1 M $CaCl_2$) was used for pH control.

Assay Procedure——Initially, 4 ml of 0.2 M Tris–HCl buffer solution and 1 ml of substrate solution were pipetted into the 10-ml reaction cell thermostated at 30 ± 0.1 °C, containing a small Teflon-coated stirring bar. The electrode was washed with water and further immersed for about 3 min in a fresh 0.1 M sodium chloride solution in order to complete the washing. Then, the old internal filling solution was replaced with a fresh 0.1 M ammonium chloride internal filling solution and the electrode was immersed in the solution. After a stable potential reading had been obtained for the stirred solution, 0.1-ml aliquots of enzyme solution were added to initiate the substrate hydrolysis. The rate of change in potential (mV/min) was calculated. The initial rate was plotted as a function of standard enzyme concentration or activity to yield a standard curve for determining the concentration or activity of other chymotrypsin samples.

Assay of Ophthalmic Chymotrypsin Preparation——Four milliliters of 0.2 M Tris–HCl buffer (pH 9.25) and 1 ml of 0.025 M *N*-benzoyl-L-tyrosinamide solution (50% ethanol) were added to the thermostated cell. After washing of the electrode as described above, the ammonia electrode was immersed in the solution and the assay procedure was started by initiating the reaction by injecting 0.1 ml of the first chymotrypsin standard into the cell. After the electrode had been washed, it was placed in the sample solution and the initial rate was measured. After another washing, the electrode was placed in the second standard, and the initial rate was measured. The enzyme activity was determined from a calibration graph of the initial reaction rate *vs.* standard chymotrypsin activity. The measurement using the spectrophotometric method[6] involves following the hydrolysis of the *N*-benzoyl-L-tyrosine ethyl ester by measurements of the extinction changes.

Results and Discussion

The basis for the enzyme-catalyzed reaction studied is the deamination of *N*-benzoyl-L-tyrosinamide.

$$\textit{N}\text{-benzoyl-L-tyrosinamide} \xrightarrow{\text{chymotrypsin}} \textit{N}\text{-benzoyl-L-tyrosine} + NH_3$$

Chymotrypsin activity is measured from the initial rate of ammonia production monitored by the ammonia gas-sensing electrode.

When the enzyme-catalyzed reaction is carried out in buffer solution containing a high concentration of the substrate, the initial rate is directly proportional to the chymotrypsin concentration or activity. *N*-Benzoyl-L-tyrosinamide as a substrate is slightly soluble in water but readily soluble in ethanol. Since a high concentration of ethanol would disintegrate the gas-permeable membrane, 0.025 M substrate solution in 50% ethanol was used. When no buffer is present, the measured electrode response (every ion-selective electrode, enzyme electrode and pH sensing gas-permeable membrane electrode[7] response) is directly proportional to the log of the concentration. On the other hand, when buffer is present the response is directly proportional to the concentration.[8] Therefore, the effects of pH were first studied to find the optimum assay conditions. The stability of chymotrypsin can be increased by calcium ions, so that in systems where addition of calcium ions does not lead to precipitation of the substrate, higher activity is measured.[2,9] Consequently, Tris–HCl buffer solution containing 0.1 M

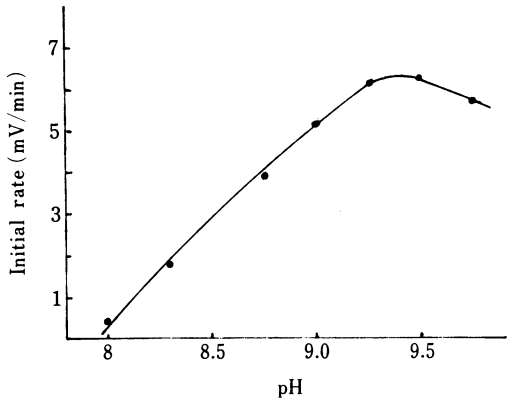

Fig. 1. Effect of pH on Hydrolysis of *N*-Benzoyl-L-tyrosinamide

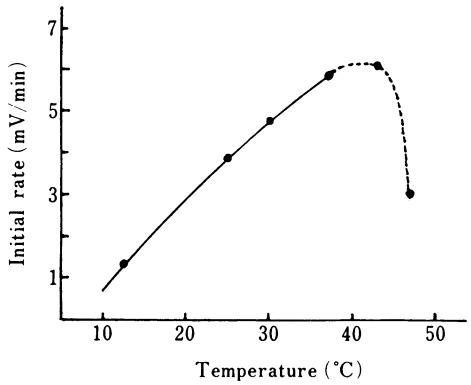

Fig. 2. Effect of Temperature on Chymotrypsin Activity

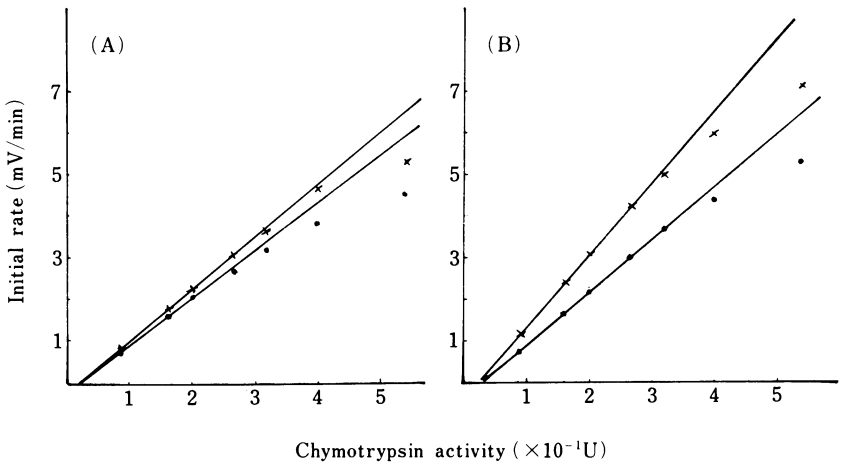

Fig. 3. Effect of Buffer Concentration on the Calibration Plot
(A) —×—, 0.1 M; —●—, 0.05 M. (B) —×—, 0.3 M; —●—, 0.2 M.

calcium chloride was employed for pH control. The optimal pH for chymotrypsin activity is pH 8,[2] but the fraction of ammonia in solution is only about 5%. On the other hand, it is about 40% at pH 9 and 100% at pH values over 11.[10] Responses of the ammonia gas-sensing electrode increased with increasing pH values, while chymotrypsin activity weakened with increasing pH value, and the initial rates consequently decreased. The initial rates of ammonia production at various pH values were monitored at 30 °C, using 0.27 U/ml chymotrypsin and 0.025 M substrate, as shown in Fig. 1. With Tris–HCl buffer containing 0.1 M calcium chloride, there was an increase in the initial rate from pH 8 to 9.5 followed by a decrease. The pH optimum for the hydrolysis of the substrate is pH 8. However, taking account of the sensitivity of the ammonia gas-sensing electrode mentioned above, reaction rates were measured at pH 9.25 instead of pH 9.5 to ensure a larger buffer capacity.

A study of the effect of temperature using the initial rate of ammonia production plotted against temperature at pH 9.25, is shown in Fig. 2. The initial rate of change in potential increased sharply with increasing temperature, but the change in potential of the electrode became unstable at temperatures over 40 °C. At a high temperature, the ethanol used as the substrate solvent evaporated into the internal filling solution of the electrode, resulting in a

TABLE I. Determination of Chymotrypsin in Aqueous Solutions

Taken (U/ml)	Number	Found (U/ml)	Recovery (%)
0.08	1	0.081	101.3
	2	0.080	100.0
	3	0.080	100.0
	4	0.080	100.0
	5	0.081	101.3
	6	0.080	100.0
			Mean 100.4
0.16	1	0.162	101.3
	2	0.163	101.9
	3	0.162	101.3
	4	0.159	99.4
	5	0.160	100.0
	6	0.161	100.6
			Mean 101.0
0.20	1	0.200	100.0
	2	0.200	100.0
	3	0.205	102.5
	4	0.203	101.5
			Mean 101.0
0.27	1	0.269	99.6
	2	0.267	98.9
	3	0.268	99.3
	4	0.267	98.9
	5	0.268	99.3
	6	0.268	99.3
			Mean 99.2

Average mean 100.4.

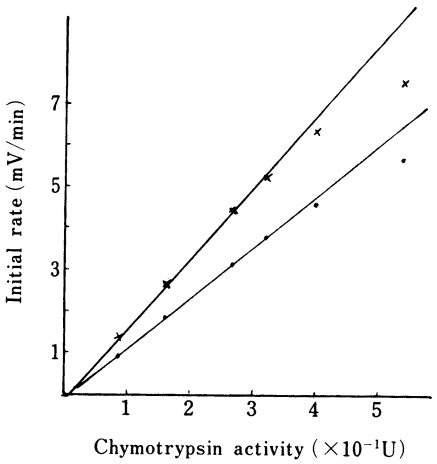

Fig. 4. Effect of pH on the Calibration Plot in 0.2 M Buffer Solution

—×—, pH 9.50; —●—, pH 9.25.

change in potential and disintegration of the gas-permeable membrane. Therefore, reaction rates were measured at 30 °C. At this temperature, where ethanol did not influence the potential and the gas-permeable membrane, stable potential responses were obtained.

The effects of buffer concentration[8] on the calibration curve using 0.025 M substrate at pH 9 and 30 °C were studied, as shown in Fig. 3. When the initial rate *vs.* the enzyme activity was plotted, a linear calibration plot was obtained in the range of 0.08—0.20 U/ml enzyme by employing 0.05 M Tris–HCl buffer solution containing 0.1 M calcium chloride. Using 0.1 and 0.3 M buffer solutions, a linear calibration plot was obtained at 0.08—0.27 U/ml enzyme. However, with 0.2 M buffer solution, a linear calibration plot was obtained at 0.08—0.32 U/ml enzyme. Based on the above data, 0.2 M Tris–HCl buffer was considered suitable for initial rate measurements.

Next, in 0.2 M Tris–HCl buffer solution, the effects of pH on a calibration plot of the initial rate *vs.* the enzyme activity using 0.025 M substrate solution at 30 °C were examined, as shown in Fig. 4. At pH 9.0 (Fig. 3B) and 9.25, a linear calibration plot was obtained at 0.08—0.32 U/ml enzyme. At pH 9.5, the slope of the calibration plot increased, but the range of the linearity was decreased.

On the basis of the above data, the most suitable experimental conditions for initial rate measurements were concluded to be pH 9—9.25 in 0.2 M Tris–HCl buffer at 30 °C. Under the optimum conditions described, chymotrypsin could be determined in the range of 0.08—0.27 U/ml of sample with an average error of 0.4% (Table I). Under the same experimental

TABLE II. Determination of Ophthalmic Chymotrypsin Activity

Number	Chymotrypsin activity (U/mg)	
	Present method	Spectrophotometric method
1	27.0	26.1
2	26.3	27.4
3	26.4	26.5
4	27.5	28.0
Mean	26.8	27.0

conditions, reaction rates may become slower because of deterioration of the glass electrode and the gas-permeable membrane. To activate the electrode, the electrode tip should be immersed in 2% ammonium fluoride for 3 min, then in 1 N HCl for several minutes, followed by exhaustive washing with water.

Similarly, changes in electrode potential caused by variation of the electrical source voltage and by electrical or electromagnetic induction effects[11] are known to occur. Therefore, in order to obtain analytical accuracy, the chymotrypsin activity[12] was determined from a calibration plot prepared for every set of determinations with reference standard enzyme solutions. In the above-mentioned experiments, *N*-benzoyl-L-tyrosinamide (0.025 M) was used as the substrate. When *N*-acetyl-L-tyrosinamide (0.025 M) was used for measurements of the initial rate of chymotrypsin activity at pH 9.25 and 30 °C, the level obtained was about 1/10 of that when the *N*-benzoyl compound was used. In order to study the applicability of the method to real samples, recovery experiments were performed using an ophthalmic chymotrypsin preparation. The crystalline preparation was diluted to bring the residual activity to the lower portion of the working range determined from reference standard enzyme solutions, as shown in Fig. 3, and the amount (units) of activity was determined from the calibration curve.[12] The accuracy of the method was further tested by comparing the results with values obtained by the spectrophotometric method.[6] Table II shows that there was satisfactory agreement between the two methods.

The sensitivities of other methods[13] for the estimation of chymotrypsin activity require microgram quantities of chymotrypsin. For instance, the spectrophotometric method with casein as a substrate requires 2—50 μg of chymotrypsin.[1] This electrode method requires 1—8 μg of chymotrypsin. The sensitivity of this method is approximately identical to the other methods, but the present method using an ammonia gas-sensing electrode offers a simple kinetic means for specific measurements of chymotrypsin activity and is advantageous because of its simplicity, accuracy, and lower cost compared with the conventional methods. Also, some organic impurities, fecal material and protein degradation products which interfere in absorption methods would not affect this electrode method.

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