Simple Automated Spectrophotometric Method for Assay of Trypsin and Chymotrypsin in Duodenal Juice

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Trypsin and chymotrypsin were automatically assayed by a simple spectrophotometric method, with specific ester derivatives as substrates. Samples containing 0.25 to 2.5 enzyme units in 0.5 ml were analyzed at a rate of 40 to 60 assays per hour, each sample being incubated for 6 min. The acidity developed during esterolysis was measured, with phenol red as the acid-base indicator. The full-scale deflection of the recorder, corresponding to 0.5 absorbance at 420 nm, was obtained with a sample containing 5 U of enzyme per milliliter, incubated in Tris-HCl buffer (25 mmol/liter, pH 8.0), in the presence of phenol red (124 μmol/liter). This variation corresponded to a decrease of 0.4 pH unit.

Additional Keyphrases: N-α-tosyl-L-arginine methyl ester as substrate for trypsin • N-acetyl-L-tyrosine ethyl ester as substrate for chymotrypsin • AutoAnalyzer • pH-stat method compared

When conveniently chosen, synthetic ester or amide substrates allow rather specific assays of trypsin (EC 3.4.1.4) and chymotrypsin (EC 3.4.2.2) in biological fluids.

An automated spectrophotometric assay has been recently proposed for trypsin (1). In this method hydrolysis of N-α-benzoyl-L-arginine ethyl ester is followed at 253 nm (2). Similar methods could easily be imagined using, for instance, N-α-tosyl-L-arginine methyl ester for trypsin (3) and N-acetyl-L-tyrosine ethyl ester for chymotrypsin assays (2). However, it is clear that all methods involving spectrophotometric measurements in the ultraviolet range are unsuitable for assays of crude physiological samples, the composition or turbidity of which yield too high and too variable absorbances at low wavelengths.

An automated trypsin assay has been proposed (4) that is based on the quantification of ammonia liberated from hydrolysis of benzoylarginine amide. However, no mention has been made of the usefulness of the method for samples other than crystallized trypsin solutions. Finally, the automated constant-pH titration previously designed in our laboratory (5) perfectly accommodates physiological samples, but the rate of analysis of 6-10 assays per hour is undesirably low.

The present paper describes a very simple spectrophotometric method that proved to be convenient for assays of trypsin and chymotrypsin in human duodenal juice, rat pancreas homogenates, and chromatographic eluates from sulfuric pancreatic extracts.

Materials and Methods

Reagents

1. Substrate solution: N-α-Tosyl-L-arginine methyl ester (TAME) and N-acetyl-L-tyrosine ethyl ester (ATEE) purchased from Serva (Heidelberg, Germany) were used for trypsin and chymotrypsin assays, respectively. Dissolve 1.895 g of TAME or 1.345 g of ATEE in 25 ml of methanol, and complete the solution with water to 100 ml to give 50 mmol/liter substrate concentrations. These solutions are stable for at least one week at 4°C.

2. Buffer-indicator solution: This solution is made of Tris-HCl (40 mmol/liter, pH 8.0) with added phenol red (0.2 mmol/liter). Dissolve 9.681 g of Tris, 142 mg of phenol red (Merck, Darmstadt, Germany), and 2 ml of "Triton X-100" (Rohm and Haas Co., Philadelphia, Pa.) in 1500 ml water. Adjust the pH to 8.0 with HCl (6 mol/liter) and dilute to 2000 ml with water.

Human Duodenal Samples

These samples were collected during 5-min intervals according to Van Der Hoeden et al. (6). The pancreatic test consisted of a combined intravenous injection, per kilogram body weight, of 1 clinical unit of secretin and 40 ng of cerulein at zero time, followed 30 min later by a second injection of 1 Ivy Unit of pancreozymin per kilogram body weight. The porcine pancreozymin-cholecystokinin and secretin we used were prepared by the Gastrointestinal Hormone Research Unit of the Karolinska Institute (Stockholm, Sweden) and cerulein was offered as the pure decapeptide by Farmitalia (Milano, Italy).

Equipment

A sampler for 400 samples (Plate SC15 with TD 15 T3 timer from Gilson Medical Electronics, Villiers-le-Bel, France) was kept at 4°C in a miniature cold room (Gilson Model RCA) in order to prevent enzyme denaturation. Fluid flow was maintained by a proportioning pump (Model I; Technicon Instruments Corp., Tarrytown, N. Y. 10591) as indicated

*Nonstandard abbreviations used: TAME, N-α-tosyl-L-arginine methyl ester; ATEE, N-acetyl-L-tyrosine ethyl ester; and Tris, tri(hydroxymethyl)aminomethane.
in Figure 1. The enzymatic reaction developed during 6 min in two consecutive jacketed mixing coils at 25°C. Forty centimeters of Teflon tubing (1.7 mm i.d.), maintained at room temperature, led to the spectrophotometer thermostated at 25°C. This spectrophotometer (Model DB-T; Beckman Instruments International S.A., 1207 Geneva, Switzerland) was equipped with a flow microcell having a 1-cm light-path (No. 178-OS; Hellma Benelux, Antwerp, Belgium) and with a Model PM 8100 recorder (Phillips, Eindhoven, The Netherlands).

Procedure

The rate of analysis was usually set at 40 samples per hour. The two sampler timers, controlling the sample probe and the liquid wash aspiration, were adjusted for 60-s sampling and 30-s washing time, respectively. Absorbance was recorded at 420 nm. The spectrophotometer was adjusted for a direct linear absorbance output from 0 to 1 A unit at 100 mV and the sensitivity of the recorder was fixed at 50 mV full scale, thus recording a maximum of 0.5 A variation.

Calibration of the Automated System

The daily calibration of the equipment included the control of the incubation time and of the colorimetric response to a given acidity. The incubation time was estimated before each series of assays, by clocking the progression of the red front of indicator, from the beginning of the first incubation coil to the flow cell. For that purpose, the tube aspirating the buffer-indicator solution was shifted to a water solution for 30 s. A small air bubble provided a sharp separation of both fluids, and the colorless front was easily located. Standards of known HCl concentration were then placed in the sampler plate. They were routinely prepared by adding 5, 10, and 15 μl of a titrated solution of 1 molar HCl to 1 ml water. As a result the colorimetric response was exactly calibrated in terms of acid microequivalents per minute of incubation, defined as enzyme units.

Blank Values

Various specimens of duodenal juice varied in their acidity and color (caused by bile pigments) (Figure 4). This difficulty was easily circumvented. Each series of assays was made in duplicate; that is, values for the samples without substrate were subtracted from those obtained with substrate.

Manual Enzymatic Assays

The assays were performed by constant pH titration as described previously (7) by use of the same substrate solutions as for the automated procedure.

Results

Validity of the Method

To check the validity of the method, an incubation medium identical to that blended by the pump in the automated system was prepared manually with TAME as substrate. Trypsin (10 U in 10 μl) was added to 3 ml of this substrate solution in a classical rectangular cell with a 1-cm optical pathway. The increase in absorbance was followed at 420 nm, with pH drop recorded simultaneously by use of a micro-electrode (Methrom EA 125; Methrom, Herisau, Switzerland). This electrode, coupled to a E-300 Methrom pH-meter, was immersed in the cell in such a way that it could not interfere with the optical beam. Figure 2 shows a plot of absorbance vs. time. The relationship was linear at least between pH 8.0 and 7.5; that is, in a pH range suitable for trypsin and chymotrypsin assays (8).

Secondly, to verify that 0.2 millimolar phenol red does not interfere with the enzymatic reactions we compared kinetic assays with and without indicator
by the "pH-stat" technique (5). No difference was observed.

Thirdly, the results of automated assays compared favorably with those for classical constant-pH titration. The samples used for comparative assays were from human duodenal juice. They were chosen because they represent the least controllable media and were therefore susceptible to give rise to the greatest errors. Each sample corresponded to a 5-min secretion period.

The values obtained for 20 trypsin assays are represented in Figure 3. Each value corresponds to the total trypsin content of a given sample, taking into account the volume recorded for each of them. The lowest values corresponded to basal secretion; the highest were obtained during pancreatic stimulation.

The values obtained with the automated colorimetric assays correlated perfectly well with the titrimetric values. The calculated coefficients of the regression line were respectively 0.975 ± 0.049 and 17.546 ± 32.403 (P > 0.99). The regression line \( y = 0.975x + 17.546 \) was thus not significantly different from the theoretical line \( y = x \).

Chymotrypsin Assays in Human Duodenal Juice

The upper part of Figure 4 shows an example of human pancreatic response to cerulein and pancreozymin represented by the tracing of chymotrypsin outflow. Nine samples were collected, each corresponding to a 5-min secretion period. Of each sample, 10 \( \mu l \) was diluted to 0.5 ml with 0.1% Triton X-100. Sample numbers 2 and 3 correspond to the stimulation and washing-out produced by the mixed secretin-cerulein injection, while samples 8 and 9 illustrate the pancreozymin effect. The lower part of Figure 4 reveals that some increase in absorbance was measured in the absence of any substrate. These measurements must be subtracted from the corresponding chymotrypsin values to provide corrected values. When corrected, these values were identical to those obtained by titration.

Discussion

The present automated method constitutes a new application of the well-documented incubating conditions already used for potentiometric methods. The only change was the introduction of 124 \( \mu \)mol of phenol red per liter in the incubation mixture, which did not affect enzymatic catalysis. The main advantages of this method are its flexibility, and the fact that it is five times as rapid as the constant-pH titration technique. This gain results from the use of single time-point assays rather than kinetic assays based on constant pH kinetics. Phenol red has already been used for the microdetermination of free fatty acids, either manually (9) or automatically (10), and for the analysis of organic acids in chromatographic column eluates (11). When buffer and indicator systems have about the same dissociation constant, the liberated acids convert the alkaline forms of buffer
and indicator into their corresponding acid forms in almost proportional amounts. The intensity of the colorimetric response depends of course on the buffer–indicator molar ratio. Although the fundamental properties applied in this new automated method are already well known, it may be useful to recall those that are of practical importance:

1. Concerning the kinetic parameters of the two enzymatic reactions, the proposed substrate concentrations are not critical. ATEE in 10 mmol/liter concentration in the incubation medium corresponds to 13 times the \( K_m \) of bovine chymotrypsin for ATEE (8), so that the rate of hydrolysis is still 95% of the initial velocity when half of the ATEE is hydrolyzed. For trypsin assays the situation is even more favorable, because a 10 mmol/liter concentration of TAME represents about 700 times the \( K_m \) for bovine trypsin. Moreover, with TAME as substrate, trypsinolysis is subject to both substrate and product activation (12, 13). The benefit of a high TAME concentration is that the assays are more sensitive and that the velocity of the reaction remains constant throughout most of the TAME hydrolysis.

2. For both enzyme assays, methanol was used to increase the solubility of the substrates. As much as 5 ml of methanol per 100 ml of incubation mixture does not affect the assay significantly (8).

3. Kinetic measurements were made at 420 nm instead of 530 nm (where sensitivity is maximum) in order to decrease baseline absorbance.

4. The products of hydrolysis (tosylarginine and acetyltirosine) are much less soluble than their respective substrates. It is therefore inappropriate to decompose more than 5 \( \mu \)mol of substrate per milliliter during the spectrophotometric assays. In fact, a somewhat prolonged incubation of a 5 millimolar tosylarginine solution results in abundant precipitation (this indicates that a 5 millimolar solution of this product is already supersaturated and remains in that state for only a few minutes).

5. Maximal velocities for trypsin and chymotrypsin catalyzed hydrolysis are obtained at a pH of about 8. Theoretically, the reaction rates decline as the pH decreases from 8.0 to 7.6. This has no substantial effect on the automated assays, as illustrated by the regression line in Figure 3. Indeed, even at high enzyme concentration when the pH of the incubation mixture gradually decreases to near 7.6, the colorimetric method gave values that were very close to those obtained by constant pH titration. The regression coefficient is 0.975. This slight deviation from the ideal value is negligible considering the degree of precision of the titration of NaOH solution by the pH-stat procedure (with standard HCl being used to calibrate the automated system).

6. The buffer strength in the useful pH range determines the sensitivity of the response.

Therefore, buffer strength must be standardized and kept constant during the corresponding assays. No problem arises for the analysis of chromatographic or electrophoretic eluates, or for pancreas homogenates with a known buffer strength. In these cases, the molarity of the Tris-HCl buffer may be increased or decreased to adjust the sensitivity of the method within large limits. The only uncertainty rests a priori in the duodenal juice analysis. However, we proved that assays of duodenal samples were equivalent by both the automated colorimetric method and the pH-stat method, which is essentially insensitive to the buffer strength of the incubates. As a consequence, a 40 mmol/liter Tris concentration is sufficient to assure constant buffering and precise results in the particular case of duodenal juice diluted approximately 200-fold in the final incubation mixture, that is, during the assays.

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References

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