A SPECTROPHOTOMETRIC DETERMINATION OF TRYPSIN AND CHYMOTRYPSIN*

by

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The observations reported in the preceding paper[1] suggested the possibility that tyrosine might exhibit pH-dependent spectral changes in the pH region in which the carboxylic acid group dissociates in addition to the well-known spectral changes which accompany the ionization of the phenolic hydroxyl group in the alkaline pH region. Investigation revealed that the technique of differential spectrophotometry could be used to detect the ionization of the carboxyl group of tyrosine.

If it is assumed that the difference spectra which are observed result from changes in the resonance of the entire molecule under the influence of changes in charge at the carboxyl position rather than from resonance differences between the ionized and unionized forms of the carboxyl group itself, it can be argued that esters of tyrosine should exhibit difference spectra relative to the carboxylate form of tyrosine which are similar to those shown by the undissociated carboxylic acid. This hypothesis was verified. Since tyrosine esters are known to be substrates for chymotrypsin[2,3], a method for the determination of chymotryptic activity was at hand.

Although it is not obvious *a priori* that similar spectral differences exist between benzoyl arginine esters and the carboxylate form of benzoyl arginine, experiment has shown this to be the case. It follows that techniques similar to those used for the estimation of chymotryptic activity can be used for the estimation of tryptic activity.

EXPERIMENTAL

Materials. L-tyrosine was a Mann product (lot number 4519). L-tyrosine ethyl ester hydrochloride (TEE·HCl) was prepared by making a concentrated solution from a commercial preparation of the free base of the ester in water as the hydrochloride at pH 5.0, removing an appreciable quantity of tyrosine by filtration, decolorizing with Norit, and taking to dryness *in vacuo*. The dry material was dissolved in absolute alcohol and recrystallized twice from absolute alcohol and ether[4]. M.p., found, 165–166°; reported[4], 166°. Nitrogen by semi-micro Kjeldahl, 5.66%; calculated, 5.70%.

N-acetyl-L-tyrosine ethyl ester (ATEE) was prepared from a portion of the purified TEE·HCl by the method of PARKS AND PLAUT[5]. M.p., found, 81°; reported[6], 79–80°.

N-acetyl-L-tyrosine (AT) was prepared by the method of DU VIGNEAUD AND MEYER[7]. M.p., found, 153°; reported[7], 152–154°.

N-benzoyl-L-arginine (BA) and N-benzoyl-L-arginine ethyl ester (BAEE) were prepared by modifications of the method of BERGMANN, FRUTON AND POLLOK[8] which have been described previously[9,10].

The trypsin and chymotrypsin used for these measurements were from a preparation which

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References p. 575.

was used extensively in earlier studies[9,11,12,13]. In view of the age of this preparation the results must be considered to illustrate a method rather than to represent properties of the enzymes.

Methods. The determinations of the difference spectra of L-tyrosine, N-acetyl-L-tyrosine and N-benzoyl-L-arginine were carried out by the technique described in the preceding paper[1].

Determinations of enzymic activity were made at 25° in the Beckman model DU spectrophotometer used for the spectral measurements. For a typical determination of chymotryptic activity 3 ml of a 0.001 *M* solution of L-tyrosine in 0.05 *M* phosphate buffer, pH 6.50, and 0.2 ml of a solution of chymotrypsin in 0.001 *M* HCl were placed in the control cuvette and the spectrophotometer was adjusted to give an optical density reading of zero with this cuvette in the light path. Three ml of a solution of 0.001 *M* TEE in the same buffer was placed in the test cuvette and at zero time 0.2 ml of the solution of chymotrypsin which had been added to the control cuvette was added to the test cuvette. Measurements of optical density were made at approximately 15 second intervals. At the completion of the reaction the control and test cuvettes gave the same optical density reading.

For the determination of trypsin this procedure was modified since BA is more absorptive than is BAEE. In this case the spectrophotometer was adjusted to give an optical density reading of zero with 0.001 *M* solution of BAEE in 0.05 *M* *tris*-hydroxymethylaminomethane in the light path. Addition of 0.2 ml of an appropriate trypsin solution in 0.001 *M* HCl caused an initial decrease in optical density, due to the dilution of the BAEE solution, and the optical density then increased linearly with time until the reading given by the test cuvette was essentially the same as that given by the control cuvette. The control cuvette contained 3 ml of 0.001 *M* BA in the same buffer as was used in the test cuvette and 0.2 ml of the same trypsin solution.

Trypsin and chymotrypsin concentrations were determined from the optical densities of their solutions at 280 and 282 mμ, respectively. The extinction coefficient used for chymotrypsin is 2.075/cm/mg/ml[13] and that for trypsin 1.71/cm/mg/ml[14].

RESULTS

The difference spectra of 0.001 *M* solutions of L-tyrosine at various pH values with reference to a 0.001 *M* solution of L-tyrosine at pH 3.07 are shown in the lower part of Fig. 1. The effect of dissociable groups other than the carboxyl is shown in the curves determined at pH 6.38 and pH 8.33. It is possible that the curve for pH 6.38 reflects the effect of dissociation of the ammonium group of tyrosine since there is little shift in the wavelength of the minimum of this curve as compared to that observed at pH 4.71 and since the p*K'* of the phenolic group of tyrosine, 10.07[15], is too high for appreciable ionization of this group to occur at pH 6.38. It is inferred, however, that the greater part of the effect shown at pH 8.33 probably results from the dissociation of the phenolic group since difference spectral measurements made on a solution at pH 9.58 against the same reference solution indicate a very high maximum in the region between 240 and 250 mμ.

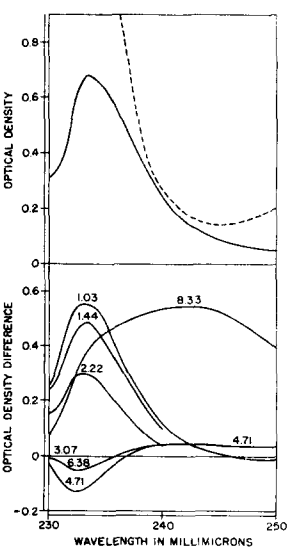

Fig. 1. Difference spectra of tyrosine and of tyrosine ethyl ester. See text.

The dashed line in the upper part of Fig. 1 shows the absolute spectrum, measured against water in the blank cuvette, of the solution L-tyrosine at pH 3.07 which was used as the reference solution for the determination of the difference spectra. The solid line in the upper part of Fig. 1 represents the difference spectrum of 0.001 *M* TEE relative to 0.001 *M* L-tyrosine, both in 0.05 *M* phosphate buffer, pH 6.50. The fact that the maximum optical density difference is nearly equal to the optical density difference between the curves shown in the lower part of the figure for pH 1.03 and pH 4.71 seems to confirm the hypothesis that TEE is analagous in its spectral behavior to undissociated tyrosine.

References p. 575.

Fig. 2 shows the variation in initial reaction velocity of the hydrolysis of TEE with chymotrypsin concentration. The reaction rate was found to decrease continuously with time, becoming zero when the optical density of the test cuvette was equal to that of the

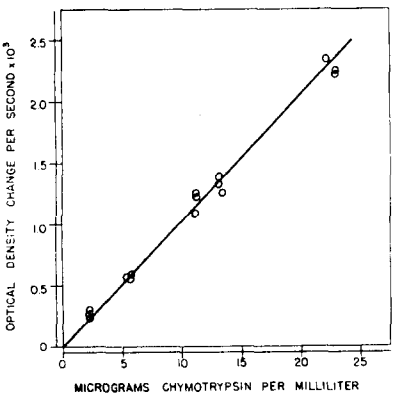

Fig. 2. Dependence of initial rate of chymotrypsin-catalyzed hydrolysis of TEE, as measured by optical density change at 233.5 mμ, upon chymotrypsin concentration at pH 6.50, 25°.

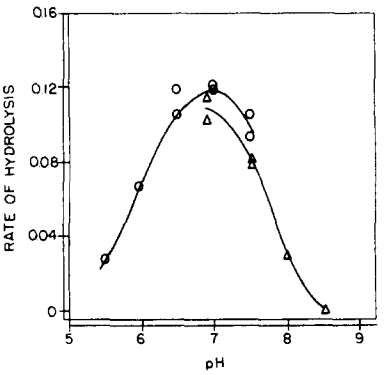

Fig. 3. Variation of initial rate of chymotrypsin-catalyzed hydrolysis of TEE with pH. Reaction rate is expressed in terms of optical density change per second × 10³ per microgram chymotrypsin per milliliter. ○ 0.05 *M* phosphate buffers; △ 0.05 *M* *tris*-hydroxymethylaminomethane buffers.

control cuvette. In a recent description of manometric assays for chymotrypsin, PARKS AND PLAUT[5] reported the evolution of carbon dioxide from bicarbonate buffer to become erratic when tyrosine began to crystallize from solutions which were initially 0.025 *M* with respect to TEE. Since the substrate solution is much more dilute in the present measurements, no interference from this source was observed.

Fig. 3 shows the pH-dependence of the observed reaction rate as measured by the present technique. The present data indicate the pH optimum to lie between 6.5 and 7.0 for this substrate. BALLS AND JANSEN[3] have reported the pH optimum to lie at pH 6.25. It is of interest that there appears to be a discontinuity in the results when 0.05 *M* phosphate buffer is replaced by 0.05 *M* *tris*-hydroxymethylaminomethane buffer, the rates being slightly slower in the *tris* buffer.

The lower part of Fig. 4 indicates the difference spectra of 0.001 *M* solutions of AT at various pH values relative to a 0.001 *M* solutions of AT at pH 3.03. It is of interest that the magnitude of the observed effects is less than half that shown in Fig. 1 for tyrosine. The dotted line in the upper part of Fig. 4 represents the absolute spectrum of the solution at pH 3.03 which was used as a reference for the measurement of difference spectra. The solid line in the upper part of

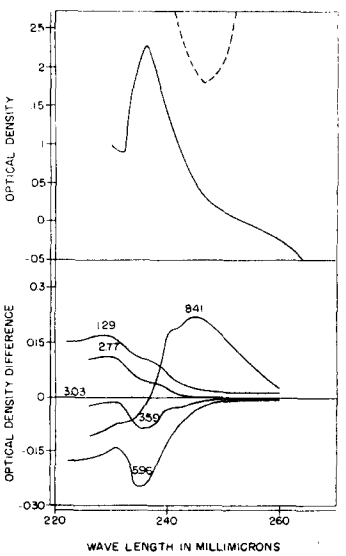

Fig. 4. Difference spectra of acetyl tyrosine and of acetyl tyrosine ethyl ester. See text for discussion.

References p. 575.

Fig. 4 shows the difference spectrum of 0.001 M ATEE in 0.05 M phosphate buffer, pH 7.00, with reference to a 0.001 M solution of AT in the same buffer. At wavelengths longer than those shown in the figure, this curve reaches a minimum at 275 mμ at an optical density difference of —0.165. Measurements of enzymic activity were made at 237 mμ to utilize the maximum optical density difference between ATEE and AT.

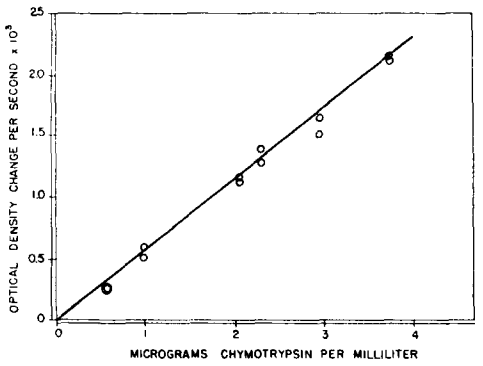

Fig. 5. Dependence of initial rate of chymotrypsin-catalyzed hydrolysis of ATEE, as measured by optical density change, upon chymotrypsin concentration at pH 7.00, 25°.

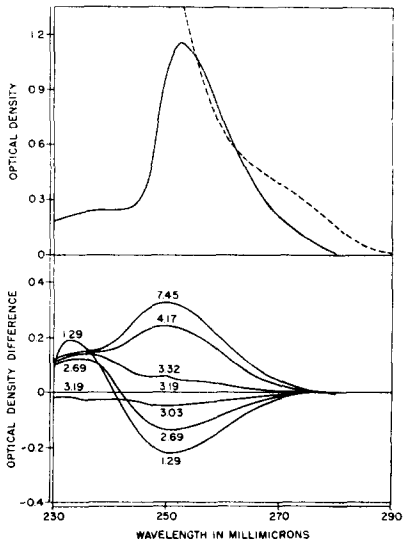

Fig. 6. Difference spectra of benzoyl arginine and of benzoyl arginine ethyl ester. See text.

The use of ATEE as a substrate is complicated by two tendencies, of opposing influence, which arise as the pH is increased beyond the range of dissociation of the carboxyl group of AT. The first of these is that the spectral differences between the carboxylic acid and carboxylate forms of AT tend to be obscured by the large absorption due to the ionization of the phenolic hydroxyl and the second is that the enzymic activity increases, becoming maximal at pH 8.2[5]. As a compromise between these opposing effects enzymic measurements were made at pH 7.00 in 0.05 M phosphate buffer.

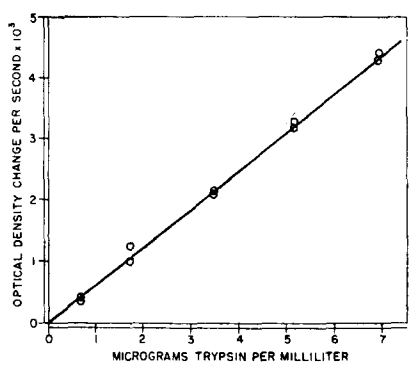

Fig. 7. Variation of initial rate of hydrolysis of BAEE by trypsin with trypsin concentration at pH 8.00, 25°.

The relation between the initial rate of optical density change and enzyme concentration is shown in Fig. 5. It is apparent that, despite the choice of a pH somewhat removed from the optimum for this enzyme-substrate pair, ATEE is still about four times as active as TEE in this assay.

The difference spectra of 0.0005 M solutions of BA at various pH values relative to a 0.0005 M solution of BA at pH 3.19 are shown in the lower part of Fig. 6. In the upper part of Fig. 6 the dashed line shows the spectrum of the reference solution which was used for the determination of difference spectra relative to water, and the solid line illustrates the spectrum of a 0.001 M solution of BA in 0.05 M phosphate buffer, pH 8.00, relative to a 0.001 M

References p. 575.

solution of BAEE in the same buffer. It should be noted that, in contrast to the behavior of the tyrosine substrates, BAEE and undissociated BA have smaller optical densities than does the dissociated form of BA.

Fig. 7 illustrates the variation of initial reaction velocity with trypsin concentration. The measurements were made at 253 mμ in 0.05 M phosphate buffer, pH 8.00. As has been previously observed[9,10] the reaction course in this case is linear until the reaction is essentially complete.

DISCUSSION

Although several methods have been devised for assaying proteolytic enzymes with specific synthetic substrates, the relatively non-specific methods, utilizing proteins as substrates, have remained the most sensitive. For example, the manometric assay for chymotrypsin reported by PARKS AND PLAUT[5] using 0.025 M L-phenylalanine ethyl ester as substrate is suitable for the determination of 60 to 600 μg of chymotrypsin and the titrimetric estimation of the esterase activity of trypsin requires quantities of trypsin of the order of 30 to 300 μg[9]. In contrast, KUNITZ' method for the assay of trypsin, using casein as a substrate, requires only 2 to 24 μg of trypsin for a determination[14]. Despite the probable partial inactivation of the enzyme preparations used in this study, it appears that the present method is suitable for the determination of quantities of chymotrypsin of the order of 1.5 to 15 μg and of quantities of trypsin ranging between 1.5 and 20 μg. Since small volumes of dilute solutions of substrates are required, this method appears to be more economical of synthetic substrates than any other available method.

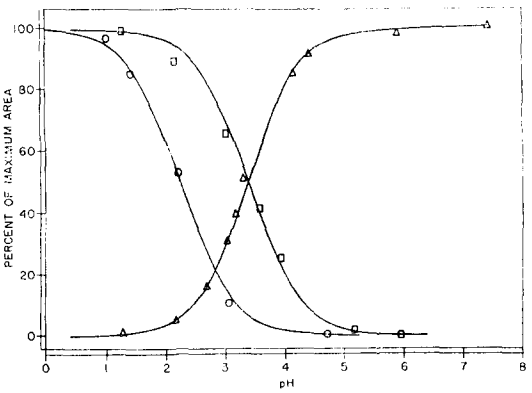

Fig. 8. Relation between the areas under difference spectral curves, expressed as per cent of maximum area, and pH. The curves are calculated from the mass law for pK' values of 2.25 for tyrosine (○), 3.40 for N-acetyl-L-tyrosine (□), and 3.40 for N-benzoyl-L-arginine (△).

The principal disadvantage to the spectrophotometric assay appears to be the requirement for a spectrophotometer equipped with a sensitive detector and with a cell compartment capable of being maintained at constant temperature.

With respect to observations incidental to the development of the assay procedures, the data shown in Figs. 1, 4, and 6 were used to estimate the apparent dissociation constants of tyrosine, AT, and BA. Measurements of the areas under the differential spectral curves were carried out by the method described in the preceding paper[1]. Areas were measured between 230 and 240 mμ from the tyrosine curves, between 232 and 244 mμ from the acetyl tyrosine curves, and between 242 and 270 mμ from the benzoyl arginine curves. The fit of the resulting measurements to calculated dissociation curves is shown in Fig. 8. The calculated curves were drawn for pK' values of 2.25, 2.30, and 3.40 for tyrosine, N-acetyl tyrosine, and N-benzoyl-L-arginine, respectively. The value of pK_1' for tyrosine is reported as 2.20[15]. Although similar measurements appear not to have

References p. 575.

been made for AT and BA, the present results are consistent with extensive measurements made on N-substituted derivatives of other amino acids[15].

The inversion of the sign of the observed spectral effects in passing from tyrosine and AT to BA appears to merit further investigation. Although no conclusion can be drawn from the present results, it is hoped that an investigation of the spectral effects found with a homologous series of phenyl aliphatic acids may reveal the basis for this effect.

SUMMARY

A sensitive spectrophotometric assay for trypsin and chymotrypsin using synthetic substrates has been described.

RÉSUMÉ

Nous avons décrit une méthode spectrophotométrique pour déterminer la trypsine et la chymotrypsine en faisant usage de substrats synthétiques.

ZUSAMMENFASSUNG

Eine empfindliche spektrophotometrische Bestimmungsmethode für Trypsin und Chymotrypsin mit Hilfe von synthetischen Substraten wurde beschrieben.

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