Trypsin

Wirnt Rick

Trypsin[1] is a proteolytic enzyme, whose precursor trypsinogen (molecular weight of both proteins is about 24000[2]) is formed in the exocrine cells of the pancreas and is secreted into the lumen of the duodenum. Trypsinogen is converted to trypsin by the enzyme enterokinase and also autocatalytically by the action of the trypsin formed. Enterokinase, which is secreted by the cells of the mucus membrane of the small intestine, acts by splitting a hexapeptide, H_2N-Val-Asp_4-Lys-COOH, from the trypsinogen[3]. The pH optimum for the reaction is between 7.0 and 9.0[1] for bovine trypsinogen. Trypsin is most stable at pH *ca.* 2.3[4]. An inert protein is also formed during the activation process[5]. This inactive protein sediments slower than native trypsin in the ultracentrifuge and has therefore been considered to be a polymer of the monomeric enzyme[6]. The formation of the inert protein can be inhibited by Ca^{2+} ions[7–9], which prevent the aggregation of the trypsin molecule[6]. Magnesium ions are inactive. Owing to the increased stability of trypsin in the presence of calcium ions, a higher activity of the enzyme is found in most assay systems when Ca^{2+} is added. However the hydrolysis of protamines and the activation of α-chymotrypsinogen are independent of Ca^{2+}[10].

The enzyme hydrolyses bonds involving the carboxyl group of lysine or arginine[11,12]. Peptides, amides and esters of these amino acids are hydrolysed and the rate increases in the following order: peptides $<$ amides $<$ esters. Proteins which are not denatured are attacked only slowly.

The following are suitable as substrates for the assay of trypsin activity: denatured proteins (haemoglobin[13], casein[14] and the esters and amides of lysine or arginine (*e.g.* *N-(a)*-benzoylargininamide[15], *N-(a)*-benzoylarginine ethyl ester[16,17], *N-(a)*-benzoylarginine-β-naphthylamide[18]).

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A. Determination with Haemoglobin as Substrate

Principle

Haemoglobin is denatured with alkaline urea solution. Trypsin hydrolyses substances from the denatured protein, which are soluble in trichloroacetic acid and whose tyrosine and tryptophan content can be determined according to the method of *Folin* and *Ciocalteu*[19]. The method of *Anson*[13] for the measurement of trypsin activity in pure solution is described below.

Optimum Conditions for Measurements

The pH optimum for the proteolytic action of trypsin is between 7.0 and 8.0. The optimum substrate concentration is around 6.7 mg. haemoglobin/ml. reaction mixture. The addition of 0.02 M Ca^{2+} prevents the formation of enzymatically inert protein (see above)[7].

Reagents

1. Bovine haemoglobin *)
2. Sodium hydroxide, A. R., 1 N and 0.5 N
3. Urea, pure, crystalline
4. Boric acid, crystalline, A. R.
5. Sodium chloride, A. R.
6. Calcium chloride solution, 5% (w/v)
7. Hydrochloric acid, A. R., 10^{-3} N, 0.2 N and *ca.* 1 N
8. Trichloroacetic acid, 5% (w/v)
9. Folin and Ciocalteu phenol reagent **)
   available ready-made, *e.g.* from E. Merck, Darmstadt, Germany.
10. L-(−)-Tyrosine, chromatographically pure.

Preparation of Solutions

I. Boric acid (1 M):
Dissolve 6.184 g. boric acid and 0.292 g. NaCl in doubly distilled water and make up to 100 ml.

II. Substrate solution:
Suspend 2.0 g. haemoglobin with about 50 ml. doubly distilled water in a 100 ml. volumetric flask, add 36 g. urea and 8 ml. 1 N NaOH, and dilute with doubly distilled water to about 80 ml. To denature the haemoglobin allow to stand for 30−60 min. at room temperature, then add 10 ml. boric acid solution (I) and after thorough shaking add 4.4 ml. 5% $CaCl_2$ solution. Adjust to pH 7.5 with 1 N HCl (glass electrode) and dilute to 100 ml. with doubly distilled water. Centrifuge off any erythrocyte stroma (15 min., 4000 g).

III. Phenol reagent:
a) The commercially available stock solution contains:
10 g. $Na_2WO_4 \cdot 2\,H_2O$, 2.5 g. $Na_2MoO_4 \cdot 2\,H_2O$, 15 g. Li_2SO_4 and 5 ml. 85% H_3PO_4 in 100 ml. *ca.* 1 N HCl.
b) Dilute solution: dilute the stock solution three-fold with doubly distilled water.

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*) *e.g.* from Behringwerke, Marburg, Germany; Armour, Kankakee, USA; Serva, Heidelberg, Germany.

**) J. biol. Chemistry *73*, 627 [1929]; see also *P. G. Hawk*, *B. L. Oser* and *W. H. Summerson*: Practical Physiological Chemistry. The Blakiston Comp., Philadelphia 1947, p. 879.

[19] *O. Folin* and *V. Ciocalteu*, J. biol. Chemistry *73*, 627 [1927].

IV. Tyrosine standard solution (10^{-3} M tyrosine):
Dissolve 181.19 mg. L-(−)-tyrosine in 0.2 N HCl and make up to 1000 ml.

Stability of the solutions

Store the substrate and tyrosine standard solution in a refrigerator at 0—4°C. To prevent the growth of micro-organisms add 2.5 mg. merthiolate*) (Lilly) per 100 ml. substrate solution, and before diluting the tyrosine solution to 1000 ml. add 14.3 ml. 35% formaldehyde (A. R.).

Procedure

Tyrosine standard curve

Pipette into 50 ml. Erlenmeyer flasks:

0.2—1.0 ml. tyrosine standard solution (IV)
4.8 ml.—4.0 ml. 0.2 N HCl
10 ml. 0.5 N NaOH.

Add with continuous shaking

3.0 ml. dilute phenol reagent (solution III b).

Measure the optical densities (as described under "Colour reaction") against a blank containing 0.2 N HCl instead of the tyrosine standard solution and plot against the μmoles tyrosine/flask.

Enzymatic reaction

For each determination prepare a blank in which the haemoglobin is first precipitated with trichloroacetic acid and then the trypsin solution (sample) is added.
Pipette into a 20 ml. centrifuge tube:

5.0 ml. substrate solution (II).

Equilibrate at 25°C in a constant temperature water bath, add

1.0 ml. enzyme solution (2—20 μg. protein in 10^{-3} N HCl),

mix and incubate for exactly 10 min. at 25°C. Add

10.0 ml. 5% trichloroacetic acid,

shake and allow to stand for 30 min. at room temperature. Filter or centrifuge off the precipitate (20 min. at 4000 g).

Colour reaction

Pipette into 50 ml. Erlenmeyer flasks:

5.0 ml. filtrate or supernatant
10 ml. 0.5 N NaOH

and with continous shaking add

3.0 ml. dilute phenol reagent (solution III b).

Centrifuge off any slight precipitate (5 min. at 4000 g). Between 5 and 10 min. after the addition of the phenol reagent, measure the optical density of the solution against distilled water in 1 cm. cuvettes at 578 mμ, 691 mμ (Photometer Eppendorf) or 750 mμ (Zeiss photometer).

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*) Sodium salt of *o*-(ethylmercurythio)benzoic acid.

Calculations

The trypsin unit (TU^{Hb}) proposed by *Anson*[13] is similar to the pepsin unit: 1 trypsin unit (TU^{Hb}) is defined as the amount of enzyme, which under the standard conditions of *Anson* (6 ml. final volume containing 0.1 g. haemoglobin, temperature: 35.5° C), hydrolyses haemoglobin at such an initial rate that the amount of hydrolysis products formed per minute has the same optical density on reaction with the phenol reagent as 1 mmole tyrosine.

The specific activity is defined as the number of trypsin units (TU^{Hb}) per mg. protein nitrogen.

At 25° C the trypsin activity is 1.78 times lower than at 35.5° C, so that the results obtained at 25° C must be multiplied by this factor. As in the case of pepsin, there is no linear proportionality between the trypsin activity and the amount of hydrolysis products formed. The trypsin activity must therefore be extrapolated to the origin or a standard curve must be used. The extrapolation method is employed for the assay of the activity of purified enzyme preparations: different and sufficiently small amounts of trypsin are incubated with the substrate as described above. After determination of the hydrolysis products (corrected for the blank) the μmoles tyrosine (obtained from the tyrosine standard curve) are plotted against the amounts of trypsin and a tangent is drawn to the curve through the origin. The amount of enzyme which liberates 1 μmole tyrosine is read off from the tangent. This amount of enzyme is multiplied by $1000 \times (5/16) \times 10 \times (1/1.78)$ to obtain the amount of enzyme corresponding to a trypsin unit. The multiplication factors are derived as follows:

1000 = conversion of μmoles to mmoles

5/16 = conversion from the colour reaction to the enzymatic reaction

10 = conversion from 10 min. to 1 min.

1/1.78 = conversion from 25° C to 35.5° C.

For routine work *Anson*[13] modified the evaluation and gave an empirical standard curve (Fig. 1).

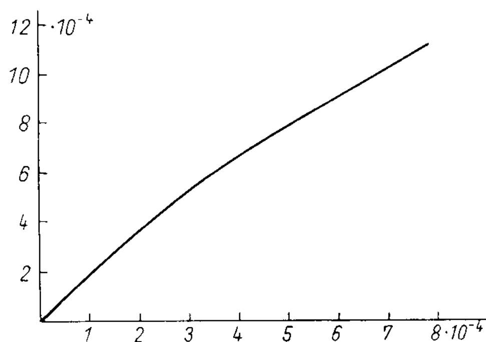

Fig. 1. Dependence of the hydrolysis of haemoglobin by trypsin on the amount of enzyme (according to *Anson*[13])

Ordinate: Tyrosine equivalent [mmoles] of the hydrolysis products in 5 ml. filtrate (see Text under "Enzymatic reaction"). Enzymatic reaction: 10 min. at 25° C.

Abscissa: Trypsin units (TU^{Hb}) according to *Anson*[13].

The optical density read on the colorimeter is converted to mmoles tyrosine by means of the tyrosine standard curve and the trypsin units corresponding to this amount are read off from the abscissa of Fig. 1. To obtain the units per ml. sample it is necessary to divide by the volume of sample taken for the assay.

Example

The standard curve prepared with L-(−)-tyrosine is linear up to 1.0 μmole/flask. The optical densities found with 1 μmole of tyrosine in the colour reaction were

at 578 mμ (Photometer Eppendorf) 0.490

at 691 mμ (Photometer Eppendorf) 0.620

at 750 mμ (Zeiss spectrophotometer) 0.665

1.75 mg. trypsin (Boehringer) in 100 ml. 10^{-3} N HCl was used. For results see Table 1.

Table 1

Trypsin [µg. per assay]	Optical density at 578 mµ	Tyrosine [µmoles in 5 ml. filtrate] *)	10^{-3} TU^{Hb} read off from Fig. 1	10^{-3} TU^{Hb} per mg. trypsin
3.5	0.060	0.122	0.065	18.6
3.5	0.065	0.133	0.070	20.0
7.0	0.124	0.251	0.135	19.3
7.0	0.133	0.272	0.145	20.7
10.5	0.198	0.404	0.220	21.0
10.5	0.198	0.404	0.220	21.0
14.0	0.263	0.537	0.310	22.1
14.0	0.258	0.527	0.300	21.4
17.5	0.327	0.668	0.400	22.8
17.5	0.315	0.644	0.375	21.8
			Mean	20.8

*) After deproteinization of the enzymatic reaction mixture with trichloroacetic acid.

One mg. trypsin is equivalent to 0.0208 TU^{Hb}. As trypsin contains 15.0% nitrogen[1], the specific activity is $0.0208 \times \frac{100}{15} = 0.139$ TU^{Hb}/mg. protein nitrogen.

Details for Measurements in Duodenal Juice

If mixtures of proteases, for example, trypsin and chymotrypsin, are assayed, the various proteolytic enzymes present will all contribute to the trichloroacetic acid-soluble peptides liberated. Only the total proteolytic activity of the duodenal juice can therefore be determined. The result can be expressed in TU^{Hb}, but it should be noted that no information can be obtained about the amounts of the different proteases.

Example

Duodenal juice (0.02 ml.) which had been centrifuged was analysed. The optical density minus the blank was 0.153, corresponding to 0.31 µmoles tyrosine. Read off from Fig. 1: 0.17×10^{-3} TU^{Hb} in 0.02 ml., corresponding to 9.5×10^{-3} TU^{Hb}/ml. duodenal juice. Consequently, the proteases contained in 1 ml. of the duodenal juice had an enzymatic activity corresponding to 8.5×10^{-3} TU^{Hb}.

B. Determination with Casein as Substrate

Principle

During the hydrolysis of casein by trypsin[14] products soluble in trichloroacetic acid are formed, and the tyrosine and tryptophan content of these is determined by measurement of the optical density at 280 mµ.

Optimum Conditions for Measurements

The pH optimum of the reaction lies between 7.0 and 8.0. As described above, calcium ions stabilize trypsin by preventing the formation of an enzymatically inactive protein from it during the incubation.

However, if calcium chloride is added to a 1% casein solution in 0.2 M borate buffer (pH 7.6)[1] to give a final concentration of 0.005 M, a slight opalescence occurs due to the formation of the insoluble calcium salt of casein[20]. In this case, the enzymatic hydrolysis is reduced by about 10% as compared to reaction mixtures without calcium ions[21] and therefore calcium is omitted from this assay.

Reagents

1. Casein according to *Hammersten* *)
2. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
3. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2\,H_2O$, A. R.
4. Trichloroacetic acid, 5%
5. Hydrochloric acid, A. R., 10^{-3} N

Preparation of Solutions

I. Phosphate buffer (0.1 M; pH 7.6):
Dissolve 0.157 g. KH_2PO_4 and 1.575 g. $Na_2HPO_4 \cdot 2\,H_2O$ in about 90 ml. doubly distilled water, adjust to pH 7.6 (glass electrode) and dilute to 100 ml. with doubly distilled water.

II. Substrate solution:
Suspend 1 g. casein in 100 ml. phosphate buffer (solution I), heat for about 15 min. in a boiling water bath until all the casein is dissolved and then dilute to 100 ml. with doubly distilled water.

Stability of the solutions

Store the buffer and substrate solution, stoppered, in a refrigerator at 0—4°C. The casein solution keeps at this temperature for at least a week.

Procedure

Spectrophotometric measurements

Wavelength: 280 mμ; silica cuvettes, light path: 1 cm.; temperature: 35°C. Measure against water. Equilibrate the substrate solution for at least 5 min. at 35°C.

Pipette successively into 12 ml. centrifuge tubes (in a 35°C water bath):

Blank 1 ml. substrate solution (II)
3 ml. 5% trichloroacetic acid.

Shake, add
1 ml. enzyme solution (sample diluted to 1 ml. with phosphate buffer, solution I) and allow to stand for 30 min. at room temperature.

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*) *e.g.* from E. Merck, Darmstadt, Germany.

20) *G. Schmidt* in *Hoppe-Seyler-Thierfelder:* Handbuch der physiologisch- und pathologisch-chemischen Analyse. 10th ed., Springer, Berlin 1960, Vol. IV, p. 627.

21) *W. Rick*, unpublished.

Experimental

0.01 – 1.0 ml. enzyme solution (sample, 1 – 25 μg. trypsin in 10^{-3} N HCl)
phosphate buffer (solution I) to 1.0 ml.
1.0 ml. pre-warmed substrate solution (II).

Mix and simultaneously start a stopwatch. After *exactly* 20 min. add

3.0 ml. 5% trichloroacetic acid

and mix thoroughly. Allow to stand for at least 30 min. at room temperature. If several determinations are to be carried out, pipette the substrate into the test tubes in the 35°C water bath at timed intervals, for example, every 30 sec. Exactly 20 min. after the start of the incubation of the first tube add 3 ml. trichloroacetic acid to each tube in the same order and at the same time interval.

Filter the contents of the blank and experimental tubes or centrifuge for 20 min. at 3000 g. Read the optical densities of the filtrates or supernatants. The optical density of the experimental tube after subtraction of the blank is used to calculate the enzyme activity.

Calculations

As the optical density of the casein hydrolysis products is not proportional to the amount of enzyme added (compare determination with haemoglobin), the initial rate of the reaction must be measured. For this, several tubes containing different amounts of sample (1 – 25 μg. trypsin) are incubated.

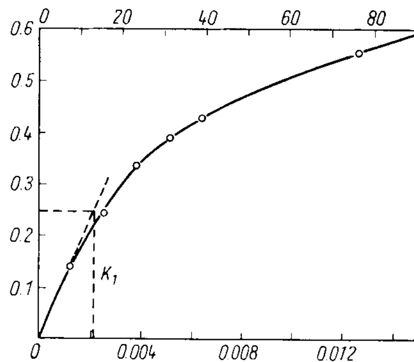

Fig. 2. Standard curve for the hydrolysis of casein by trypsin (according to *Kunitz*[14]). Volume of the incubation mixture: 2 ml.; casein concentration: 0.5%; 20 min. incubation at 35°C; volume after the addition of trichloroacetic acid: 5 ml.; final concentration of trichloroacetic acid: 3%.

Ordinate: Optical density of the hydrolysis products liberated from casein in 20 min. by the action of trypsin (1 cm. light path; 5 ml. final volume).

Lower abscissa: mg. trypsin in 1 ml. incubation mixture.
Upper abscissa: 10^{-3} TU^{Cas} in 1 ml. incubation mixture.

Plot the optical densities against the μg. enzyme/ml. incubation mixture (for a 2 ml. incubation mixture divide the amount of trypsin per tube by 2) (Fig. 2). Draw a tangent to the initial part of the curve; the slope of this is a measure of the specific activity of the preparation.

According to *Kunitz*[14] a unit (TU^{Cas}) is the amount of trypsin which under the defined conditions (20 min. incubation at 35°C, final volume of the incubation mixture: 2.0 ml., after addition of

trichloroacetic acid: 5 ml.) liberates sufficient trichloroacetic acid-soluble hydrolysis products, so that the optical density at 280 mμ increases by 1.00 in 1 min. Specific activity: TU^{Cas}/mg. protein.

According to Fig. 2

$$TU^{Cas}/\mu g. \text{ trypsin} = \frac{0.25}{2.1 \times 20} = 6 \times 10^{-3}, \textit{ i.e. } 1 \text{ mg. crystalline trypsin contains } 6\ TU^{Cas},$$

where

0.25 = ΔE_{280}/20 min.
2.1 = μg. trypsin/ml.
20 = conversion of ΔE/20 min. to ΔE/min.

The upper abscissa of Fig. 2 gives the TU^{Cas} corresponding to the ordinate (ΔE_{280}/20 min.). This scale is independent of the purity of the enzyme preparation. The activity of unknown samples can be read directly from the curve (Fig. 2) in TU^{Cas}.

The optical factor[14], *i.e.* the reciprocal of the optical density of a solution of 1 mg. protein/ml. at 280 mμ and 1 cm. light path serves to determine the protein concentration of the enzyme solution. For crystalline trypsin the optical factor is 0.585[14].

Example

"Boehringer" trypsin (9.84 mg.) was dissolved in 10 ml. 10^{-3} N HCl and diluted with phosphate buffer to 100 ml. Optical density at 280 mμ: 0.166; protein content: $0.166 \times 0.585 = 0.097$ mg./ml. The sample solution was diluted 1:10 with phosphate buffer (solution I) and 0.1 to 1.0 ml. was analysed. The optical density for 0.1 ml., corresponding to 0.485 μg. protein/ml. assay mixture, was 0.056. The curve in Fig. 2 is still a straight line at this optical density. Therefore the calculations can be made directly with the formula given on p. 802 and hence:

$$\frac{0.056 \times 10^3}{0.485 \times 20} = 5.8\ TU^{Cas}/\text{mg. protein.}$$

10^3 = conversion of μg. to mg. protein.

Details for Measurements in Duodenal Juice

As casein is also hydrolysed by chymotrypsin the determination is not specific for trypsin. Therefore only the total proteolytic activity of the duodenal juice can be measured with casein as substrate.

Example

ml. duodenal juice:	0.005 ml.	0.01 ml.
Optical density		
Experimental	0.503	0.930
Blank	0.045	0.057
Difference	0.458	0.873
Calculations[22]	$\frac{0.458}{0.005 \times 20} = 4.58\ TU^{Cas}$/ml.	$\frac{0.873}{0.01 \times 20} = 4.37\ TU^{Cas}$/ml.

As both amounts give practically the same activity per ml., the use of smaller volumes of sample is unnecessary. The standard curve (Fig. 2) is approximately linear over this range, therefore the formula on p. 802 is used for the calculations.

[22] *W. Rick* in *A. Gigon* and *H. Ludwig:* Enzymatische Regulationen in der Klinik. Schwabe, Basle and Stuttgart 1961, p. 251.

C. Determination with Benzoylarginine Ethyl Ester as Substrate

Principle

N-(α)-Acylarginine esters have been used as substrates for trypsin[16,17]. The reaction was followed by direct electrometric titration of the carboxyl groups liberated[16]. *Schwert* and *Takenaka* found that the absorption of *N-(α)*-benzoyl-L-arginine ethyl ester is much weaker at 253 mμ than that of *N-(α)*-benzoyl-L-arginine, hence the hydrolysis of the ester can be followed spectrophotometrically[17]. In the absence of thrombin, plasmin and kallikrein the method is specific for trypsin[11,12].

Optimum Conditions for Measurements

The pH optimum of the hydrolysis is at 8.0[17]. The rate is maximal with $> 1.5 \times 10^{-4}$ M substrate[23]. *N-(α)*-benzoyl-L-arginine does not inhibit[16]. With substrate concentrations above 3×10^{-4} M the reaction is linear until about 90% of the substrate has been consumed[23]. Calcium chloride is added to a final concentration of 0.02 M because of its stabilizing action on trypsin.

Reagents*)

1. Tris-hydroxymethyl-aminomethane, tris
2. *N-(α)*-Benzoyl-L-arginine**)
3. *N-(α)*-Benzoyl-L-arginine ethyl ester hydrochloride**)
   chromatographically pure
4. Calcium chloride solution, 5% (w/v)
5. Hydrochloric acid, A. R., 10^{-3} N; 2 N

Preparation of Solutions

I. Tris buffer (0.05 M; pH 8.0):
Dissolve 6.057 g. tris in about 900 ml. doubly distilled water, adjust to pH 8.0 (± 0.05) (glass electrode) with *ca.* 15 ml. 2 N HCl and dilute to 1000 ml. with doubly distilled water.

II. Substrate solution (0.001 M):
Dissolve 34.29 mg. *N-(α)*-benzoyl-L-arginine ethyl ester hydrochloride in about 90 ml. buffer (solution I), add 4.44 ml. 5% $CaCl_2$ solution and dilute with buffer (solution I) to 100 ml.

III. *N-(α)*-Benzoyl-L-arginine (0.001 M):
Dissolve 27.83 mg. *N-(α)*-benzoyl-L-arginine in buffer (solution I) and make up to 100 ml.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 0–4° C. About 4% of the ester (solution II) is hydrolysed spontaneously[23] in 24 hours. This is not important as long as the substrate concentration in the assay mixture is above 1.5×10^{-4} M. Consequently, the solution is stable for at least 14 days at 4° C. The absorption of solution III does not change in 14 days.

Procedure

Spectrophotometric measurements

Preliminary remarks: The difference spectrum of *N-(α)*-benzoyl-L-arginine ethyl ester and *N-(α)*-benzoyl-L-arginine shows a sharp maximum at 253–254 mμ[17]. In this laboratory

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*) Complete reagent kits are available commercially, see p. 1037.
**) *e.g.* from Mann Research Laboratories, New York 6, N. Y., USA.
23) *W. Rick*, unpublished.

the maximum was found to be at 254 mμ[23]. As the absorption decreases rapidly on either side of the maximum, it is important that measurements are made with a narrow band-width. If the Zeiss spectrophotometer PMQ 2 is used, it is possible with an amplification of I/10/10 and a slit-width of 0.25 mm., to set the optical density of the ester solution to 0. The corresponding half band-width for 254 mμ is then about 0.9 mμ. Because of the narrow maximum of the difference spectrum small errors in setting the wavelength cause considerable variation in the molar extinction coefficient and consequently errors in the calculations. It is therefore advisable to check the wavelength scale with a mercury lamp. To calibrate the wavelengths the following mercury lines are used: 244.6, 246.4, 248.2, 257.6, 260.3, 264.0 and 265.2 mμ. The assays are especially elegant and accurate, if the measurements of optical density can be made with a continuous recording spectrophotometer.

Method: Wavelength: 254 mμ; silica cuvettes, light path: 1 cm.; final volume: 3.2 ml.; temperature: 25°C (constant temperature cuvette holder). Measure against the blank.

Pre-warm the substrate solution to 25°C in a water bath.

Pipette successively into the cuvettes:

Blank

3.0 ml. substrate solution (II)
0.2 ml. doubly distilled water.

Place in the spectrophotometer and adjust to zero optical density with the smallest possible half band-width (about 1 mμ).

Experimental

3.00 ml. substrate solution (II)
0.19—0 ml. buffer (solution I)
0.01—0.20 ml. enzyme solution (sample).

Mix with a plastic paddle, start a stopwatch and read the optical density against the blank every 30 sec. for 2—4 min.

The optical density difference ΔE/min. should not be greater than 0.400, otherwise dilute the enzyme solution 1 : 2 or 1 : 4 with buffer (solution I).

To check the substrate concentration measure the optical density of solution III against solution II. The optical density difference should be larger than 0.5.

Calculations

Schwert and *Takenaka*[17] defined the activity of their enzyme preparation in (ΔE/sec.)/mg./ml. reaction mixture. Over the range tested there was proportionality between ΔE/sec. and the amount of enzyme added up to a ΔE/sec. of 0.005.

As in contrast to the methods requiring natural substrates a defined reaction is measured with this method, the activity of the enzyme can be expressed in μmoles of substrate converted/min. according to the proposal of *King*[24] and *Webb*[25]. The specific activity is defined as the amount of substrate reacting (μmoles/min.) per mg. protein or per ml. body fluid.

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[24] *E. J. King* and *D. M. Campbell*, Proc. 4th. Internat. Congr. on Clinical Chemistry, Edinburgh 1960. E. & S. Livingstone, Edinburgh and London 1961, p. 185; *E. J. King* and *D. M. Campbell*, Clin. chim. Acta *6*, 301 [1961].

[25] *E. C. Webb*, Proc. 4th. Internat. Congr. on Clinical Chemistry, Edinburgh 1960. E. & S. Livingstone, Edinburgh and London 1961, p. 55.

According to[23,26] the optical density difference between 10^{-3} M solutions of *N-(a)*-benzoyl-L-arginine and the ethyl ester is 1.15 at 25°C, pH 8.0 and with a 1 cm. light path. With a volume of 1 ml. an optical density change of +1.15/min. corresponds to the conversion of 1 μmole of substrate/min. With a 3.2 ml. assay volume the reaction of 1 μmole of substrate/min. is equivalent to an increase in optical density of 0.359/min. The activity is therefore calculated according to the following formula:

$$\frac{\Delta E_{254}/\text{min.}}{0.359} = \mu\text{moles substrate hydrolysed/min.}$$

If the activity is divided by the mg. protein or the ml. duodenal juice per reaction mixture, the specific activity is obtained.

Example

Trypsin (3.78 mg.) [recrystallized twice, salt-free (Serva)] was dissolved in 10 ml. 10^{-3} N HCl. The optical density of the solution at 280 mμ and with a 1 cm. light path was 0.640; the protein concentration was 0.640 × 0.585 = 0.374 mg./ml.

ml. sample		0.05 ml.	0.10 ml.
mg. trypsin/reaction mixture		0.0187	0.0374
Optical density at 254 mμ after	0 sec.	0.062	0.120
	10 sec.	0.120	0.220
	20 sec.	0.164	0.330
	30 sec.	0.214	0.420
	40 sec.	0.256	0.510
	50 sec.	0.306	0.600
	60 sec.	0.351	0.690
ΔE_{254}/min.		0.289	0.570

$$\text{Specific activity } \frac{0.289}{0.359 \times 0.0187} = 43.1\ \mu\text{moles/min./mg.}$$

$$\frac{0.570}{0.359 \times 0.0374} = 42.5\ \mu\text{moles/min./mg.}$$

Example of the measurement of trypsin activity in duodenal juice

Refer to [27]. 0.05 ml. duodenal juice was analysed.

Time [min.]	Optical density [254 mμ]	ΔE/0.5 min.
0	0.260	
		0.051
0.5	0.311	
		0.049
1	0.360	
		0.044
1.5	0.404	
		0.046
2	0.450	
		0.062
2.5	0.512	
		0.046
3	0.558	
		0.048
3.5	0.606	
		0.046
4	0.652	
	Mean	0.049/0.5 min.

[26] *I. Trautschold* and *E. Werle*, Hoppe-Seylers Z. physiol. Chem. *325*, 48 [1961].
[27] *G. Lundh*, Scand. J. clin. Lab. Invest. *9*, 229 [1957].

The optical density increase per min. is 0.098

Calculations: $\frac{0.098}{0.359 \times 0.05} = 5.46$ µmoles/min./ml. duodenal juice

Stability of the Enzyme in Duodenal Juice

Duodenal juice should be collected in tubes which are cooled in ice water. There is no decrease in trypsin activity in 7 hours if the samples are stored at 0 – 4° C [27]. If it is necessary to keep the material for longer periods, it should be stored frozen at – 15 to – 20° C.

Sources of Error

To check the specificity of the reaction with *N*-(*a*)-benzoyl-L-arginine ethyl ester [12,27] 190 µg. chymotrypsin per reaction mixture was used. The optical density change per minute was 0.0039 and therefore the activity of the preparation was $\frac{0.0039}{0.359 \times 0.190} = 0.057$ µmoles/min./mg. protein.

The ratio of the specific activities of trypsin and chymotrypsin is 43 : 0.057 or 754 : 1. It is possible that the slight activity of the chymotrypsin preparation was due to contamination.