Chymotrypsin

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In addition to trypsin, chymotrypsin also occurs in duodenal juice. This enzyme not only hydrolyses protein, but also coagulates the casein of milk. Two inactive precursors, α-chymotrypsinogen and chymotrypsinogen B, have been isolated from bovine pancreas[1]. Both precursors are activated by trypsin. The first step in the activation of α-chymotrypsinogen is the formation of π-chymotrypsin. If the activation is carried out rapidly (large amounts of trypsin) π-chymotrypsin is converted autocatalytically to δ-chymotrypsin. However, if the activation proceeds slowly (small amounts of trypsin) autocatalytic hydrolysis of π-chymotrypsin will lead to the formation of α-chymotrypsin with the release of two dipeptides, seryl-arginine and threonyl-asparagine[2,3]. On prolonged storage, β and γ-chymotrypsin and an enzymatically inactive protein are formed autocatalytically from α-chymotrypsin. If α-chymotrypsin is incubated for an hour at 35°C, only γ-chymotrypsin is formed. Chymotrypsinogen B is activated by trypsin to chymotrypsin B. The stability of chymotrypsin is increased by calcium ions, resulting in a higher activity being obtained in their presence[4]. The molecular weight of α-chymotrypsinogen is around 23000, and that of α-chymotrypsin around 22000. The different forms of the enzyme can be distinguished by means of their electrophoretic mobility, behaviour on chromatography on ion exchange resins, their crystal form, *etc.* α-Chymotrypsin and chymotrypsin B have the same substrate specificity but hydrolyse different synthetic substrates at different rates.

All the enzymes of this group hydrolyse proteins and peptides, but in addition they also hydrolyse esters and amides of the aromatic amino acids[5,6]. In the case of proteins, peptide bonds involving the carboxyl groups of aromatic amino acids such as phenylalanine and tyrosine are hydrolysed especially easily. The reaction rate decreases in the following order: esters (especially *N*-acyl tyrosine esters) > amides > proteins. Esters of tryptophan, methionine, norvaline and norleucine, and also *N*-benzoyl-L-arginine methyl ester are hydrolysed at much slower rates.

Proteins (haemoglobin, casein[7]) and synthetic substrates[5] (*e.g.* *N*-(α)-benzoyl-L-phenylalanine-β-naphthyl ester[8], L-tyrosine ethyl ester, *N*-acetyl-L-tyrosine ethyl ester[9], *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester[9a]) are suitable as substrates for the assay of the activity of chymotrypsin. The activity of pure chymotrypsin solutions, for example, in the purification of the enzyme, can be determined with haemoglobin (see under Trypsin, p. 808).

A. Measurements with Casein as Substrate

Principle

Casein is hydrolysed by chymotrypsin with the formation of hydrolysis products, whose tyrosine and tryptophan content can be measured spectrophotometrically after precipitation of the residual substrate. Apart from minor variations the method corresponds to that described for the assay of the activity of trypsin (see p. 811).

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1) *M. Laskowski* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 8.
2) *M. Dixon* and *E. C. Webb:* Enzymes. Longmans, Green & Co., London 1958, p. 548.
3) *F. Turba* and *W. Zillig* in *K. F. Bauer:* Ergebnisse der medizinischen Grundlagenforschung, Thieme, Stuttgart 1960, Vol. III, p. 253.
4) *N. M. Green, J. A. Gladner, L. W. Cunningham* jr. and *H. Neurath*, J. Amer. chem. Soc. *74*, 2122 [1952].
5) *H. Neurath* and *G. W. Schwert*, Chem. Revs. *46*, 69 [1950].
6) *M. Dixon* and *E. C. Webb:* Enzymes. Longmans, Green & Co., London 1958, p. 252.
7) *F. C. Wu* and *M. Laskowski*, J. biol. Chemistry *213*, 609 [1955].
8) *H. A. Ravin, P. Bernstein* and *A. M. Seligman*, J. biol. Chemistry *208*, 1 [1954].
9) *G. W. Schwert* and *Y. Takenaka*, Biochim. biophysica Acta *16*, 570 [1955].
9a) *C. J. Martin, J. Golubow* and *A. E. Axelrod*, J. biol. Chemistry *234*, 294 [1959].

Optimum Conditions for Measurements

The pH optimum for the hydrolysis of casein by chymotrypsin is 8.0[1]. The optimum substrate concentration is obtained with a final concentration of 0.4% casein[10]. The addition of calcium chloride (0.005 M) to the substrate solution increases the activity of crystalline α-chymotrypsin by 10–12%[4]; this concentration is optimal.

Reagents

1. Casein according to *Hammarsten*
2. Boric acid, crystalline, A. R.
3. Sodium hydroxide, A. R., 1 N
4. Hydrochloric acid, A. R., 1 N; 0.1 N; 10^{-3} N
5. Calcium chloride, 5% (w/v)
6. Trichloroacetic acid, 5% (w/v)

Preparation of Solutions

I. Borate stock solution (0.8 M):
Suspend 49.5 g. boric acid in doubly distilled water, dissolve by the addition of 400 ml. 1 N NaOH and dilute to 1000 ml. with doubly distilled water.

II. Borate buffer (0.1 M; pH 8.0):
Add about 130 ml. doubly distilled water to 25 ml. borate solution (I), adjust to pH 8.0 ± 0.05 (glass electrode) with 1 N and 0.1 N HCl and dilute to 200 ml. with doubly distilled water.

III. Substrate solution:
Suspend 1 g. casein in 95 ml. 0.1 M borate buffer (solution II), heat the mixture in a boiling water bath (about 10 min.) until the casein has dissolved, add 1.1 ml. 5% $CaCl_2$ solution and dilute to 100 ml. with buffer (solution II).

Stability of the solutions

The substrate solution keeps for about 1 week in a refrigerator at 0–4° C if stored stoppered.

Procedure

See under "Trypsin", p. 812.
Dissolve the chymotrypsin in 10^{-3} N HCl and use 2–50 μg. enzyme per assay. Use borate instead of phosphate buffer.

Calculations

A unit analogous to that defined by *Kunitz*[11] for trypsin can be used (see, for example, α-chymotrypsin in[12]). Fig. 1 (from[1,7]) shows the relationship between the optical density of the reaction mixture and the amounts of α-chymotrypsin and chymotrypsin B added. Under the conditions described here, α-chymotrypsin is more active than chymotrypsin B. For example, in Fig. 1 a concentration of 6 μg. α-chymotrypsin per ml. assay mixture gives an optical density of 0.60; the number of units (for measurements with casein as substrate) is therefore $\frac{0.60}{0.006 \times 20} = 5.0\ \text{units}^{\text{Cas}}/\text{mg. protein}$.

[10] *W. Rick*, unpublished.
[11] *M. Kunitz*, J. gen. Physiol. *30*, 291 [1947].
[12] Biochemica-Katalog, C. F. Boehringer & Soehne G. m. b. H., Mannheim, Germany, edition 1960.

The protein concentration of the enzyme solution is determined with the optical factor as in the case of trypsin, *i.e.* with the reciprocal of the optical density of a solution of 1 mg. protein per ml. at 280 mμ and 1 cm. light path. The optical factor for α-chymotrypsin is 0.500[1].

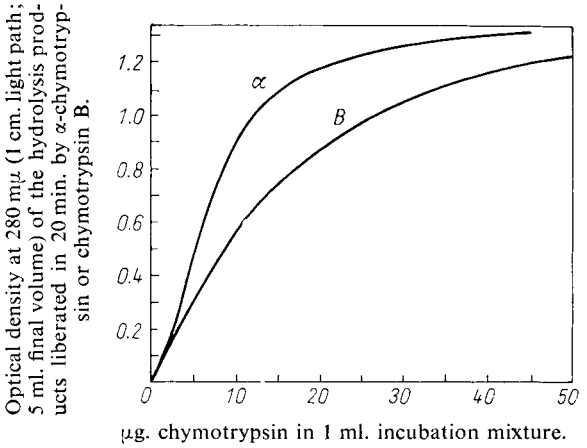

Fig. 1. Standard curves for the hydrolysis of casein by α-chymotrypsin and chymotrypsin B. (according to[1]). Volume of the incubation mixture: 2 ml.; casein concentration: 0.5%; incubation: 20 min. at 35°C; volume after the addition of trichloroacetic acid: 5 ml.; final concentration of trichloroacetic acid: 3% (w/v).

Example

"Boehringer" α-chymotrypsin (9.00 mg.) was dissolved in 10^{-3} N HCl and made up to 100 ml. The optical density of the solution at 280 mμ was 0.176, therefore the concentration of the solution was $0.176 \times 0.500 = 0.088$ mg. protein/ml. Protein concentration after dilution 1:10 with 10^{-3} N HCl: 8.8 μg./ml. This solution (0.1—1.0 ml.) was analysed. The optical densities (ordinate) obtained were plotted against the amount of enzyme per assay mixture (abscissa). An optical density of 0.124 was obtained for 1 μg. chymotrypsin/ml. 0.5% casein solution. Therefore the specific activity is:

$$\frac{0.124 \times 10^3}{1.0 \times 20} = 6.2 \text{ units}^{Cas}\text{/mg. protein}$$

where

0.124 = ΔE_{280}/20 min.

10^3 = factor to convert from μg. to mg.

1.0 = μg. chymotrypsin/ml.

20 = factor to convert from 20 min. to 1 min.

Details for Measurements in Duodenal Juice

See under "Trypsin", p. 814, for details and example.

B. Determination with L-Tyrosine Ethyl Ester as Substrate[9]

Principle

If the carboxyl group of tyrosine is not dissociated, the tyrosine absorbs more strongly between 230 and 240 mμ than if carboxyl group is dissociated. Tyrosine ethyl ester behaves like undissociated tyrosine, so that the hydrolysis of the ester can be followed by measurements of the absorption. By

plotting the optical density against time a reaction curve is obtained, from which it is easy to determine the initial rate of the reaction (refer to p. 35). Trypsin does not hydrolyse tyrosine ethyl ester, so that in studies on pancreatic enzymes the method is specific for chymotrypsin.

N-Acetyl-L-tyrosine ethyl ester (optimum pH 8.2) is attacked considerably faster than L-tyrosine ethyl ester[9]. However, the conditions for the measurements with the *N*-acetyl derivative are less favourable. The measurements cannot be made in the optimum pH range, because the differences in the absorption spectrum of the ester and the free acid are smaller between pH 6 and 8 due to the very strong absorption resulting from the dissociation of the phenolic OH group[9].

Optimum Conditions for Measurements

The pH optimum for chymotrypsin in this assay is 7.0[9]. The maximal rate of the reaction is not reached with 2×10^{-3} M substrate[10]. However, at this substrate concentration the hydrolysis of the tyrosine ethyl ester, and therefore the decrease in optical density, is linear until 20% of the ester has reacted. As α-chymotrypsin is stabilized by calcium ions the substrate solution contains 0.02 M Ca^{2+}. Consequently, the phosphate buffer is replaced by tris buffer, although the enzyme is slightly more active in the former[9].

Reagents

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

Preparation of Solutions

I. Tris buffer (0.05 M; pH 7.0):
Dissolve 6.057 g. tris-hydroxymethyl-aminomethane in about 900 ml. doubly distilled water, adjust to pH 7.0 ± 0.05 (glass electrode) with *ca.* 23 ml. 2 N HCl and dilute to 1000 ml. with doubly distilled water.

II. L-Tyrosine (0.001 M):
Dissolve 18.12 mg. L-tyrosine in buffer (solution I) and make up to 100 ml.

III. L-Tyrosine ethyl ester (0.002 M; 0.02 M Ca^{2+}):
Dissolve 49.14 mg. L-tyrosine ethyl ester in about 90 ml. buffer (solution I), add 4.44 ml. 5% $CaCl_2$ solution and dilute to 100 ml. with buffer (solution I).

Stability of the solutions

Store the buffer and substrate solution in a refrigerator at 0–4° C. About 4% of the substrate hydrolyses spontaneously in 24 hours. The optical density of the substrate solution should exceed 0.65 (conditions of measurement see below).

Procedure

Spectrophotometric measurements

Preliminary remarks: The maximum of the difference spectrum of tyrosine and tyrosine ethyl ester is at 234 mμ[10]. For these measurements a Zeiss quartz monochromator M 4 Q II

*) *e.g.* from Mann Research Laboratories, New York 6, N. Y., USA.

with a slit-width of 0.075 mm. was used (half band-width of 0.22 mμ). If a slit-width of 1.5 mm. (analogous to[13]) is used, the maximum is displaced to 232 mμ and is 12% lower than the maximum at 234 mμ. The extinction coefficient at 234 mμ remains constant with 0.15—1.1 mμ half band-width (corresponding to 0.05—0.4 mm. slit-width). If the amplification I/10/10 on the indicating device is not sufficient, switch to II. With this it is possible to set the optical density of the blank (containing 3 ml. tyrosine solution, 0.15 ml. water and 0.05 ml. duodenal juice) to 0 (slit-width 0.4 mm.).

The difference spectrum shows a narrow absorption band[9]. Although the measurements are made at the maximum of the band, it is recommended that a check be made of the agreement between the monochromator and the wavelength scale. A mercury lamp *) is suitable for this purpose, since the lines it emits at 225.9, 232.3, 234.5, 235.8, 237.7 and 240.0 mμ can be used to standardize the monochromator[10].

Method: Wavelength: 234 mμ; silica cuvettes, light path: 0.5 and 1 cm.; final volume: 1.6 and 3.2 ml. respectively; temperature: 25°C (constant temperature cuvette holder).

Pre-warm the tyrosine and tyrosine ethyl ester solution (II and III) in a water bath at 25°C.

Blank

Pipette successively into a 1 cm. cuvette and mix:

3.00 ml. tyrosine solution (II)
0.19—0.10 ml. doubly distilled water
0.01—0.10 ml. enzyme solution (sample).

Place in a spectrophotometer and set to an optical density of 0 using the smallest possible half band-width (about 0.7—0.8 mμ, corresponding to a slit-width of 0.25—0.3 mm.).

Experimental

Pipette successively into a 0.5 cm. cuvette and mix:

1.50 ml. tyrosine ethyl ester solution (III)
0.09—0 ml. doubly distilled water
0.01—0.10 ml. enzyme solution (20—60 μg. enzyme sample).

Mix with a plastic paddle, start a stopwatch and measure the optical density against the blank every 10—15 sec. for 2—4 min. Plot the optical densities against the time and from this curve obtain the initial rate of the reaction (ΔE/min.). Generally, the optical density decrease is linear in the first minute after the addition of enzyme.

Calculations

According to[9,13] the measured values can be expressed in (ΔE/sec.)/mg./ml. The standard curve published by *Schwert* and *Takenaka*[9] gives optical density differences from 0.0002—0.0023 per sec. for about 2—22 μg. crystalline chymotrypsin per ml. reaction mixture (indicating linear proportionality in this range between the amount of enzyme added and the amount of ester hydrolysed).

Like the hydrolysis of benzoylarginine ethyl ester (see "Trypsin", p. 814), the hydrolysis of tyrosine ethyl ester by chymotrypsin is a defined reaction. It is therefore possible to express the activity in

*) *e.g.* St 40, Quarzlampengesellschaft, Hanau, Germany.
[13] *G. Lundh*, Scand. J. clin. Lab. Invest. *9*, 229 [1957].

μmoles substrate reacting/min. according to the proposals of *King*[14] and *Webb*[15]. The specific activity is then defined as the μmoles substrate reacting/min./mg. protein or per ml. body fluid.

According to[9] the optical density difference at 234 mμ (1 cm. light path) between 10^{-3} M solutions of tyrosine and tyrosine ethyl ester is 0.672. If the volume is 1 ml., then an optical density change of 0.672/min. indicates the reaction of 1 μmole of substrate/min.; with an assay volume of 3.2 ml., an optical density difference of 0.672/3.2 = 0.210/min. corresponds to the hydrolysis of 1 μmole of substrate/min. The activity is therefore calculated according to the formula:

$$\frac{\Delta E_{234}/\text{min.}}{0.210} = \mu\text{moles substrate hydrolysed/min.}$$

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

Example

"Boehringer" α-chymotrypsin (3.44 mg.) was dissolved in 10 ml. 10^{-3} N HCl. 0.2 ml. of this solution was taken for assay (0.0688 mg. chymotrypsin/reaction mixture).

Time [sec.]	Optical density
0	0.562
10	0.546
20	0.528
30	0.509
40	0.489
50	0.472
60	0.457
ΔE_{234}/min.	0.105

$$\text{Activity: } \frac{0.105}{0.210 \times 0.0688} = 7.26 \text{ μmoles/min./mg.}$$

Example of the measurement of trypsin activity in duodenal juice

Refer to[13]. Used: 0.05 ml. duodenal juice. Owing to the strong absorption of the juice the amplification of the spectrophotometer was increased, so that the optical density was reduced by 0.200.

Time [sec.]	Optical density
0	0.418
10	0.400
20	0.382
30	0.360
40	0.340
50	0.320
60	0.306
ΔE_{234}/min.	0.112

$$\text{Activity: } \frac{0.112}{0.210 \times 0.05} = 10.7 \text{ μmoles substrate hydrolysed/min./ml.}$$

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

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

Therefore the amount of enzyme contained in 1 ml. duodenal juice hydrolyses 10.7 μmoles tyrosine ethyl ester/min. Accordingly it is equivalent to

$$\frac{10.7}{7.26} = 1.47 \text{ mg. } \alpha\text{-chymotrypsin.}$$

Stability of the Enzyme in Duodenal Juice

If the duodenal juice is collected in tubes cooled in ice and is stored at 0—4° C, then in the range examined, no decrease of chymotrypsin activity occurs up to 7 hours after collection[13]. If the samples are to be assayed later than 7 hours after collection, it is recommended that they be stored at −15° to −20° C.

Sources of Error

Trypsin does not interfere since it does not hydrolyse tyrosine ethyl ester[13].