Pepsin, Pepsinogen, Uropepsinogen

Wirnt Rick

The proteolytic enzyme pepsin is found in the gastric juice of all vertebrates. The central or chief cells of the gastric mucosa secrete the inactive precursor pepsinogen (molecular weight of pepsinogen from pig stomach: 42500), which on cleavage of a peptide inhibitor (mol. wt. 3242) and other neutral peptides (mol. wt. about 1000), is converted to the proteolytically active pepsin (mol. wt. 34500)[1]. The activation (pH <5.0) is an autocatalytic process[2].

The specificity of pepsin is not yet fully known[3]. The enzyme splits peptide bonds, but not amino acid esters or amides. Bonds in which the amino group of phenylalanine or tyrosine and the carboxyl group of glutamic acid are concerned are especially easily hydrolysed[4]. In insulin bonds between leucine and valine are also hydrolysed[5].

Methods of determination[6]: Viscosity measurements with protein solutions; determination of the nitrogen content of the trichloroacetic acid-soluble compounds liberated; formol titration of the amino groups formed after the action of the enzyme on gelatine, casein, edestin, milk proteins, *etc.*[7]; determination of the tyrosine and tryptophan content of the hydrolysis products with the phenol reagent of *Folin* and *Ciocalteu*[8] after the action of the enzyme on haemoglobin[9,10]; determination of the rennin activity of the enzyme[11,12,19]; release of labelled material from isotopically labelled serum albumin[13]; decrease of ribonuclease activity after the action of pepsin[14].

Principle

Pepsin hydrolyses fragments from denatured haemoglobin which are soluble in trichloroacetic acid[10,15]. The tyrosine and tryptophan content of these substances is determined according to *Folin* and *Ciocalteu*[8]. The method of *Anson*[10] is described below for the measurement of pepsin activity in gastric juice.

Optimum Conditions for Measurements

The optimum pH for proteolytic activity is around 1.5—2.5[16] or 1.8—2.0[15,17]. However, the hydrolysis of carbobenzoxy-L-glutamyl-L-phenylalanine is optimal at pH 4.5 and of carbobenzoxy-L-glutamyl-L-tyrosine at pH 4.0[4]. According to *Ohlenbusch*[17] the pH activity curve of pepsin is

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15) *R. M. Herriott* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 6.
16) *E. L. Smith* in *J. B. Sumner* and *K. Myrbäck:* The Enzymes. Academic Press, New York 1951, Vol. I, p. 840.
17) *H. D. Ohlenbusch*, Lecture, Meeting of the French, Swiss and German Societies for Physiological Chemistry, Zürich 1960.

contributed to by the change in activity with pH below pH 2 and the change in activity due to denaturation of substrate above pH 2. With urea-denatured haemoglobin the pH optimum is 3.1 and at pH 4 not less than 50% of the activity at the pH optimum is obtained (denaturation of the haemoglobin in 40% alkaline urea, enzyme reaction in 10% urea solution). The optimum substrate concentration is 1.6 mg. haemoglobin/ml. and the rate of hydrolysis is constant between 1.6 and 16 mg. substrate/ml.

Reagents

1. Hydrochloric acid, conc. (36% w/w) *)
2. Hydrochloric acid, A. R., 0.2 N
3. Hydrochloric acid, A. R., 0.06 N
4. Hydrochloric acid, A. R., 0.01 N
5. Sodium hydroxide, A. R., 0.5 N
6. Trichloroacetic acid
7. Sodium tungstate, $Na_2WO_4 \cdot 2H_2O$ *)
8. Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$ *)
9. Lithium sulphate, Li_2SO_4 *)
10. Orthophosphoric acid, conc. (85% w/w) *)
11. Bovine haemoglobin **)
12. L-(−)-Tyrosine
    chromatographically pure.

Preparation of Solutions

I. Substrate solution:
Dissolve 2 g. haemoglobin in 0.06 N HCl and make up to 100 ml. (pH *ca.* 1.8). Centrifuge off any stromata which may be present at 4000 g. for 15 min.

II. Phenol reagent +):
This reagent can be obtained commercially, *e.g.* from E. Merck, Darmstadt, Germany, If it is wished to prepare the phenol reagent, the best procedure is that of *E. Layne* +).

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

IV. Trichloroacetic acid (5% w/v):
Dissolve 5 g. trichloroacetic acid in distilled water and make up to 100 ml.

Stability of the solutions

Store the substrate and tyrosine solutions in a refrigerator at 0 to 4° C. To prevent bacterial contamination 2.5 mg. Merthiolate++) (Lilly) can be added per 100 ml. substrate solution and formaldehyde (0.5% final concentration) to the tyrosine solution.

*) The reagents 1, 7, 8, 9 and 10 are components of the *Folin* and *Ciocalteu* phenol reagent[8]. This reagent can be obtained commercially, *e. g.* from E. Merck, Darmstadt, Germany.

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

+) *E. Layne* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 448.

++) Na salt of *o*-(ethylmercurythio)-benzoic acid

Procedure

Tyrosine standard curve

Pipette into 50 ml. Erlenmeyer flasks:

0.2—1.0 ml. tyrosine standard solution (III) (= 0.2—1.0 μmoles tyrosine)
4.8—4.0 ml. 0.2 N HCl
10.0 ml. 0.5 N NaOH.

Add with continual shaking

3.0 ml. dilute phenol reagent (solution II).

Measure the optical density as described below against a blank containing 0.2 N HCl instead of tyrosine standard and plot the results against μmoles tyrosine.

Enzymatic reaction

For each determination prepare a blank: add 10 ml. trichloroacetic acid (IV) to 5 ml. of the substrate solution (I), then add the enzyme solution, and after a short time filter or centrifuge off the precipitate (see below).

Pipette into a 20 ml. centrifuge tube:

5.0 ml. substrate solution (I).

Warm to 25° C in a constant temperature bath, either add

0.01—0.04 ml. undiluted gastric juice and
0.99—0.96 ml. 0.01 N HCl

or mix in

1.00 ml. enzyme solution (containing 5—20 μg. crystalline protein).

Incubate at 25° C. After exactly 10 min. add

10.0 ml. trichloroacetic acid solution (IV)

and shake.

Filter or centrifuge off the precipitate (20 min. at 4000 g).

Colour reaction

Pipette into a 50 ml. Erlenmeyer flask:

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

Add with constant shaking

3.0 ml. dilute phenol reagent (solution II)

and 5 to 10 min. after the addition of the phenol reagent read the optical density against water in 1 cm. cuvettes at 578, 691 or 750 mμ.

Calculations

The "Pepsin Unit" (PU^{Hb}) defined by *Anson*[10] is the amount of enzyme which hydrolyses haemoglobin at such an initial rate under the standard conditions (total volume 6 ml. containing 0.1 g. haemoglobin; temperature 35.5° C), that the amount of trichloroacetic acid-soluble hydrolysis products formed per minute, gives the same optical density with the phenol reagent as 1 mmole

tyrosine. If the incubation is carried out at 25°C instead of 35.5°C, then the activity of the enzyme is 1.82 times lower. However, there is no linear proportionality between the pepsin activity and the amount of hydrolysis products formed. Therefore the pepsin activity must be extrapolated to zero or a standard curve can used.

The *extrapolation method* is used for the determination of activity of purified enzymes: different, sufficiently small amounts of enzyme are incubated with the substrate as previously described. After determination of the hydrolysis products (corrected for the blank) the μmoles tyrosine are plotted against the amounts of enzyme and then a tangent to the curve is drawn through the origin. From the tangent the amount of enzyme which corresponds to 1 μmole tyrosine is read off. This amount of enzyme is multiplied by

$$10 \times 1\,000 \times \frac{5}{16} \times \frac{1}{1.82} = 1717$$

to give the amount of enzyme corresponding to one pepsin unit (PU^{Hb}).

The factors are obtained as follows:

10 = conversion from 10 min. to 1 min.
1000 = conversion from μmoles to mmoles
5/16 = conversion from colour reaction to enzymatic reaction mixture
1/1.82 = conversion from 25°C to 35.5°C

For studies on gastric juice and other biological fluids *Anson*[10] has given a *standard curve* obtained by use of a modified assay (Fig. 1).

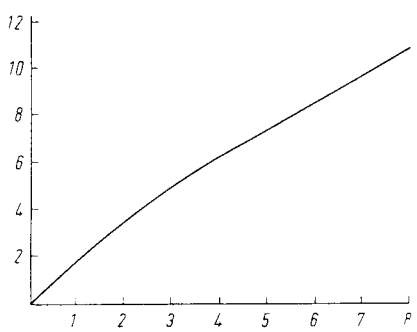

Fig. 1. Dependence of the hydrolysis of haemoglobin by pepsin on the amount of enzyme (according to *Anson*[10])

Ordinate: Tyrosine equivalent [mmole × 10^4] of the hydrolysis products in 5 ml. filtrate (see text under "Enzymatic reaction"). Enzymatic reaction: 10 min. at 25°C.

Abscissa: Pepsin units according to *Anson*[10], $PU^{Hb} \times 10^4$.

The optical density read on the spectrophotometer is converted to mmoles tyrosine by means of the tyrosine standard curve and the pepsin units PU^{Hb} corresponding to this amount are read off from the abscissa of Fig. 1. For conversion to units per ml. sample the results must be divided by the volume of sample taken for the assay.

Calculation of the results in International Units:

The activity of 1 PU^{Hb} at 25°C is $\frac{1.000}{1.82}$ = 549.4 μmoles of tyrosine-equivalents liberated/min.

Example

The standard curve prepared with L-(−)-tyrosine is a straight line up to 1 μmole tyrosine/mixture. The optical densities found with 1 μmole tyrosine (volume: 18 ml.) were

at 578 mμ	0.490
at 691 mμ	0.620
at 750 mμ	0.665

0.04 ml. gastric juice from a healthy subject were used for the enzymatic reaction. After the colour reaction the optical density at 578 mμ (minus the blank) was 0.186. This corresponds to 0.38 μmoles tyrosine. According to Fig. 1 this value corresponds to 2.18×10^{-4} PU^{Hb} (0.04 ml. gastric juice). Consequently the gastric juice contained $2.18\times10^{-4}\times25 = 5.45\times10^{-3}$ PU^{Hb} pepsin/ml.

Measurement of Pepsinogen Activity

Pepsin is destroyed at pH >8 [15], while pepsinogen is stable at this pH. Therefore it can be determined in the presence of pepsin, if the solution containing the enzyme and pro-enzyme is brought to pH 8 with NaOH, followed by acidification to pH 2–3 with HCl to convert the pepsinogen to pepsin. Further operations as for pepsin.

Measurement of Uropepsinogen Activity

A small fraction of the pepsinogen formed by the gastric mucosa is secreted into the blood and excreted as uropepsinogen in the urine [18, 19]. To convert uropepsinogen into pepsin acidify the urine to pH 2.5–3 with HCl and incubate for 1 hour at 37°C [12, 19]. Further operations as for pepsin.

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