TRYPSIN

Prepared at the 15th JECFA (1971), published in NMRS 50B (1972) and in FNP 52 (1992). An ADI 'not limited' was established at the 15th JECFA (1971)

SOURCES

Derived from purified extracts of porcine or bovine pancreas

Active principles

Trypsin (serine proteinase)

Systematic names and numbers

None (EC 3.4.21.4)

Reactions catalyzed

The enzyme hydrolyzes polypeptides, amides and esters, especially at linkages involving the carboxylgroups of L-arginine or L-lysine yielding peptides of lower molecular weight.

DESCRIPTION

White to tan amorphous powders ; soluble in water and practically insoluble in alcohol, chloroform and ether

FUNCTIONAL USES

Enzyme preparation
Used in baking, tenderizing of meat and the production of protein hydrolysates

GENERAL SPECIFICATIONS

Must conform to the *General Specifications for Enzyme Preparations used in Food Processing* (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

Trypsin activity

The sample shows proteolytic activity
See description under TESTS

TESTS

IDENTIFICATION TESTS

Trypsin activity

Principle of Method:
Denatured hemoglobin is digested under standard conditions, the undigested hemoglobin is precipitated with trichloroacetic acid, and the amount of unprecipitated protein split products, which is a measure of the amount of proteinase present, is estimated with phenol reagent, giving a blue colour with tyrosine and tryptophane.

Reagents
- 0.2 N Hydrochloric acid
- 0.1 N Hydrochloric acid
- 1 M Potassium dihydrogen phosphate
- Sodium chloride solution, 1%
- 0.5 N Sodium hydroxide solution
- 1.0 N Sodium hydroxide solution
- 0.3 N Trichloroacetic acid

- Urea
- Beef blood, whipped
- Formaldehyde, 0.15%
- Merthiolate (Lilly)
- Phenol reagent: to the phenol reagent prepared according to Folin and Ciocalteau (Folin, O. and V., Ciocalteau, J. Biol. Chem., 73, 627, (1927) twice its volume of water is added)
- Tyrosine

Procedure

The Preparation of Hemoglobin

Whipped beef blood is centrifuged 20-30 min. The serum and the white corpuscles which form a thin layer on top of the red corpuscles are siphoned off and the red corpuscles are then mixed with an equal volume of cold 1% sodium chloride solution and after centrifugation the supernatant solution is siphoned off again and the corpuscles are either stored frozen or dialyzed immediately and then stored frozen. The corpuscles are largely freed of colour producing substances not precipitable by trichloroacetic acid by dialysis in Du Pont cellophane tubing of 3/4 in diameter. Cellophane deteriorates on standing, especially the outer layer of the coil which is most exposed to the air. It is necessary to test the tubing for leaks. One end of the tubing is wetted with water and a knot is tied in the end of the tubing. The tube is then filled with water and the open end is twisted and folded over. While the folded part is pressed closed with one hand the tubing is squeezed with the other. The squeezing, in addition to showing up leaks, stretches the tubing and thus excessive stretching and dilution during dialysis are avoided. If the tubing is satisfactory the water is poured out, a marble is put in, the tube is filled with the washed corpuscles and the end of the tubing is closed with a knot in the cellophane itself. The tubes are placed in a tall vessel. Cold tap water is run into the lower part of the vessel at a rate sufficient to cause stirring. Occasionally the tubes are inverted and the hemoglobin solution is thus stirred by the marbles.

After 24 h dialysis the hemoglobin solutions from all the cellophane tubes are mixed and the mixed solution is stored frozen in small aluminium containers or cardboard ice cream containers. It is easily possible to prepare enough dialyzed hemoglobin at one time for thousands of proteinase estimations.

To estimate the concentration of protein in the dialyzed corpuscles a 3-5 g sample is weighed out in a porcelain evaporating dish, dried overnight at 105°, and the dry weight recorded. The number of grams of protein per cubic cm of sample is:

$$\frac{W_p}{(W_s - W_p) + 0.73 \times W_p}$$

where

W_p = weight of dried protein

W_s = weight of sample

When it is not convenient to store the dialyzed hemoglobin frozen it can be stored at room temperature as a dry powder. If the hemoglobin solution is frozen while the drying takes place the hemoglobin remains soluble. Bacto-hemoglobin of the Difco brand can be used for rough work when the blank is not of importance. It consists of dried washed corpuscles. The other commercial hemoglobin, are prepared from unwashed corpuscles or from whole blood. Some of them give results very different from those obtained with the hemoglobin whose preparation has just been described. Cassin and edestin or egg albumin can be used instead of hemoglobin. Gelatin cannot be used since it is not precipitated by trichloracetic acid.

Phenol Reagent

To the phenol reagent prepared according to Folin and Ciocalteau (1927) twice its volume of water is added. Whenever the phenol reagent is referred to this diluted reagent is meant.

The Estimation of Trypsin

(Hemoglobin Substrate)

A solution is made up containing 8 ml of 1 N sodium hydroxide, 72 ml water, 36 g urea, and 10 ml of 22% hemoglobin (22 g hemoglobin per 100 ml solution). This alkaline solution is kept at 25^{o} for 30-60 min to denature the hemoglobin and is then mixed with a solution containing 10 ml of 1 M potassium dihydrogen phosphate and 4 g of urea. The final pH is 7.5. 1 mg merthiolate (Lilly) is added to each 50 ml of hemoglobin solution as a preservative. The hemoglobin solution is stored at 5^{o} and is stable for weeks.

Smaller or larger quantities of substrate solution can, of course, be made up so long as the components are added in the manner and the proportions given.

Four ml of 2.5% hemoglobin solution is added with an automatic pipette to a 175 x 20 mm test tube. Then 1 ml of 0.3 N hydrochloric acid is added from an automatic pipette. The final pH is 1.6. The acid substrate solution is stored at 5^{o} and used within a day or two since in some cases the blank increases with time. If the substrate solution is kept for more than an hour before being used, the test tube should be stoppered.

Digestion and Colour Development

Digestion is carried out at 25^{o}. A convenient holder for the tubes is a piece of wood or bakelite with holes slightly larger than the diameter of the tubes. The tubes float upright in the bath. Enzyme and substrate are brought to the digestion temperature before digestion is begun.

1 ml of enzyme solution is added to 5 ml of substrate solution and the two solutions are mixed by whirling the tube. After 10 min, 10 ml of 0.3 N trichloroacetic acid (estimated by titration) is added, the tube is shaken vigorously, and the suspension is filtered after 30 min.

A filter paper such as Whatman No. 3 which does not absorb split products must be used. The colour value of the split products should be the same whether the protein precipitated by trichloroacetic acid is removed by filtration on centrifugation.

To 5 ml of the digestion filtrate in a 50 ml Erlenmeyer flask are added 10 ml of 0.5 N sodium hydroxide and 3 ml of the phenol reagent. The solution is whirled during the addition of phenol reagent. Since the colour formed depends somewhat on the rate at which the phenol reagent is added this rate is standardized by adding the reagent as rapidly as is possible and still have the reagent come out of the burette as drops. The colour is read against the standard after 2-10 min.

The Standard

The standard tyrosine solution contains 0.0008 meq of tyrosine (0.112 mg tyrosine nitrogen) in 5 ml 0.2 N hydrochloric acid, with 0.5% formaldehyde as a preservative. The concentration of tyrosine is determined by the Kjeldahl method. Five min are allowed for the colour development after the addition of 10 ml of 0.5N sodium hydroxide and 3 ml of the phenol reagent in the manner already described.

In practice a blue glass is used as a standard instead of the blue solution obtained from 0.0008 meq tyrosine. To avoid the necessity of finding a blue glass which exactly matches the colour of the tyrosine-phenol reagent solution a No. 241 Corning glass filter is placed in or above the eye piece of the colorimeter. In the fairly monochromatic red light transmitted by this filter different blues look alike. The blue glass standard is calibrated with the tyrosine standard and this calibration is frequently checked.

Blank

10 ml of 0.3 N trichloroacetic acid is mixed with 5 ml of hemoglobin solution, 1 ml of enzyme solution is added, the tube is again shaken, and the suspension is filtered after 30 min. When a purified enzyme solution is used for digestion the blank is not increased by the enzyme solution and 10 ml of 0.3 N trichloroacetic acid is added to a mixture of 5 ml hemoglobin solution and 1 ml water. 1 ml of tyrosine solution containing 0.0008 meq of tyrosine dissolved in 0.1 N hydrochloric acid containing 0.5% formaldehyde is added to 5 ml of the blank trichloroacetic acid filtrate. The colour is then developed by the addition of 10 ml sodium hydroxide and 3 ml phenol reagent and read after 5 min against the standard.

Calculations

Colour value of 5 ml digestion filtrate in meq tyrosine =

$$\frac{CR_s}{CR_{df}} \times 0.0008$$

where
CR_s = colorimeter reading for standard
CR_{df} = colorimeter reading for digestion filtrate

Colour value of 5 ml blank filtrate =

$$\frac{CR_s}{CR_{bf+tyr}} \times 0.0008 \times 19 - 0.0008$$

where
CR_{bf+tyr} = colorimeter reading for blank filtrate + added tyrosine
Unless split products are added with the enzyme the blank is usually about 0.00008 meq of tyrosine.

Colour value of digestion products in 5 ml of digestion filtrate = colour value of 5 ml of digestion filtrate - colour value of 5 ml blank filtrate.
The number of activity units corresponding to the colour value of the digestion products in 5 ml of digestion filtrate is read off from the curve (Fig. 1). When the blank is constant one can omit the calculation of colour values and use a curve in which the colorimeter reading for 5 ml digestion filtrate is plotted against activity units.