Amylase

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Amylases are found in almost all plants, animals and micro-organisms. Especially large amounts of amylase occur in the pancreas of higher animals, and in the saliva of man, ape, pig, rat, guinea-pig, mouse and squirrel[1]. Smaller amounts of amylase are found in serum, urine, liver and muscle. The saliva of carnivorous animals contains no amylase.

Animal amylase is mainly α-amylase; β-amylases occur in plants. β-Amylase attacks starch at the non-reducing ends and splits off β-maltose. It hydrolyses starch to a limit dextrin and its action is thought to be arrested by the 1,6-cross-linkages. α-Amylase attacks 1,4 linkages which lie within the starch molecule, liberating first dextrins and then a mixture of reducing sugars (especially α-maltose).

There are four main types of method for the determination of amylase activity:

1. Determination of the residual substrate by precipitation with ethanol-water. This method is not readily applicable to serum, because the proteins are also precipitated.
2. Measurement of the decrease in viscosity of the incubation mixture during the enzymatic hydrolysis. This method depends considerably on the type of substrate and the reproducibility of the results is very poor.
3. Measurement of the amount of reducing sugar liberated by the enzyme[2–5]. This method depends on the ratio of amylose to amylopectin in the substrate, which often varies with the source. It can also give misleading results with serum when the blood sugar level is above 150 mg./100 ml.[5a].
4. Measurement of changes in the iodine colour of the assay mixture[6–11]. Such a method (*Street* and *Close*[11]) is described below.

There is also a turbidimetric method[12, 13] and one which depends on the adsorption of congo red on starch[14].

Principle

The solution to be examined is allowed to act on amylose for 15 min. and then the blue colour formed on addition of a iodine-iodide solution is compared with the colour of an amylase-free control.

Optimum Conditions for Measurements

The pH activity curve is fairly flat between pH 6.9 and 7.1; pH 7.0 is most suitable for measurements. Animal amylase is activated by chloride ions. In the method described here maximum activity is obtained when the chloride concentration in the enzymatic reaction mixture is >0.01 N. A chloride content of up to 0.9% does not interfere. Plant amylase is not activated by chloride ions.

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A linear relationship between the amylase activity and the colour of the iodine reaction is obtained up to an amylase content in the sample of 60 Street-Close units*)/100 ml. under the conditions described here (15 min. incubation at 37° C). With higher concentrations of amylase the colour produced by the iodine is no longer blue, but instead may be violet, red or yellow[11].

Reagents

1. Amylose
   obtained from starch (see Appendix, p. 858) or commercial preparation (*e.g.* British Drug Houses Ltd. Poole, Dorset, England).
2. Potassium dihydrogen phosphate, KH_2PO_4
3. Disodium hydrogen phosphate, Na_2HPO_4
4. Hydrochloric acid, A. R., 0.01 N
5. Iodine, sublimed
6. Potassium iodide, A. R.
7. Sodium chloride, A. R.
   a) 0.1 N solution for the micromethod
   b) 0.85% (w/v) solution for the ultramicromethod
8. Sodium hydroxide, A. R., 0.1 N
9. Ethanol

Preparation of Solutions

Prepare all solutions with glass distilled water and, with the exception of the iodine solution, store in polyethylene containers.

I. Amylose**)
   a) Stock solution (1% w/v):
   Heat 80 ml. 0.1 NaOH to 90° C in a 250 ml. beaker, remove the flame and pour into this solution a suspension of 1.0 g. amylose in 5 ml. ethanol. Rinse traces of amylose from the container and stirring rod with two 1 ml. portions of ethanol and add the washings to the hot NaOH. Most of the ethanol boils off immediately. Cool the solution, transfer to a volumetric flask and dilute to 100 ml. with 0.1 N NaOH. Mix well and store at room temperature.
   b) Dilute solution (0.1% w/v):
   Dilute 10 ml. of the stock solution in a volumetric flask to 100 ml. with distilled water and store at room temperature.

II. Phosphate buffer (0.02 M; pH 7.0):
   Dissolve 3.471 g. Na_2HPO_4 + 2.118 g. KH_2PO_4 in distilled water and make up to 2000 ml.

III. Iodine-potassium iodide solution
   a) Stock solution (0.1 N iodine):
   Dissolve 30 g. KI in 250 ml. distilled water. Dissolve 12.7 g. iodine in this solution and dilute to 1000 ml. with distilled water. Store in the dark.

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*) According to[12] 100 Street-Close units are contained in 100 ml. of sample, when 0.1 ml. (1 ml. 1 : 10 diluted solution) hydrolyses 2 mg. amylose in 15 min. at pH 7.0 and 37° C.

**) If the amylose solutions have to be filtered use sintered glass filters, because paper absorbs amylose.

b) Dilute solution (0.01 N iodine):
Dilute 10 ml. of the stock solution to 100 ml. with distilled water. Store in the dark.

Procedure

Experimental material

The method described here is only suitable for the determination of amylase in serum. If the pH optimum of plant and bacterial amylases is known and the buffer is modified accordingly, this method can also be used for the estimation of these enzymes. Dilute serum samples 1 : 10 with 0.1 N NaCl solution (0.2 ml. serum + 1.8 ml. 0.1 N NaCl) and 1 : 40 (0.2 ml. serum + 7.8 ml. 0.1 N NaCl).

Enzymatic reaction

Pipette into three 100 ml. volumetric flasks:

	Test 10	*Test 40*	*Standard*
phosphate buffer (solution II)	5 ml.	5 ml.	5 ml.
amylose solution (Ib)	2 ml.	2 ml.	2 ml.
0.01 N HCl	2 ml.	2 ml.	2 ml.

Place the volumetric flasks marked "Test 10" and "Test 40" in a beaker of water in a water bath at 37°C for 3 min. (contents of the flasks equilibrate to 37°C). Add to "Test 10"

1 ml. 1 : 10 diluted serum

and to "Test 40"

1 ml. 1 : 40 diluted serum.

Allow the flasks to stand exactly for 15 min. in the 37°C water bath. Dilute the contents of all three flasks to about 80 ml. with distilled water. Quickly add

4 ml. iodine solution (IIIb),

dilute to 100 ml. with distilled water and mix thoroughly *). Measure the optical density of the solutions at 620 mμ (red filter, *e.g.* Ilford filter No. 204) against distilled water.

Calculations

The 1 : 10 dilution of serum allows the determination of from 0 to 35 Street-Close units/100 ml. serum. If, after addition of the iodine solution, the contents of the flask "Test 10" are not blue-green, but violet, reddish or yellow, then use the optical density of the "Test 40" mixture for the calculations. If this dilution is also not blue-green, then repeat the estimation using a greater dilution of serum (1 : 100 or 1 : 150).

The amylase concentration in the serum is calculated from the following formula:

Dilution 1 : 10

$$\frac{100}{E_S}(E_S - E_T) = \text{Street-Close units/100 ml. serum}$$

Dilution 1 : 40

$$\frac{400}{E_S}(E_S - E_T) = \text{Street-Close units/100 ml. serum}$$

where

E_S = optical density of the standard
E_T = optical density of the test.

*) Thorough mixing is essential for the success of the determination.

Ultramicrodetermination

Close and *Street*[15] have modified their micromethod[11] in the following way to allow the ultramicrodetermination of serum amylase:

Reagents and solutions

As for the micromethod (see p. 855). Immediately before use mix:

IV. Buffered substrate solution:
5 ml. phosphate buffer (solution II) + 2 ml. 0.01 N HCl +
2 ml. amylose solution (Ib) + 1 ml. 0.85% (w/v) NaCl solution.

Experimental material

Allow capillary blood from a heel or finger prick to run into a glass tube 10 cm. long (outer diameter: 3 mm., inner diameter: 1 mm., one end tapering to an outer diameter of 1 mm.). Almost fill the tube, seal the non-tapered end with sealing wax or in a flame. Place the tube on a cotton wool pad inside a test tube and centrifuge. Break the tube at the junction of the serum and cells and allow serum to run directly into a pipette. For a detailed description of this procedure, see[16–18].

Enzymatic reaction

Pipette into a test tube (15 cm. long, about 1.25 cm. diameter) with a ground-glass stopper:

0.1 ml. 0.85% (w/v) NaCl solution

and place in a water bath at 37° C. Allow

10 µl. of serum

to run in from a micropipette*). Mix in

1 ml. buffered substrate solution (IV, warmed to 37° C),

start a stopwatch, stopper tube and incubate for exactly 15 min. at 37° C. Then add:

10 ml. distilled water
0.6 ml. iodine solution (IIIb),

mix thoroughly and measure the optical density at 620 mµ (see micromethod). The following mixture serves as a standard:

1 ml. buffered substrate solution (IV)
0.1 ml. 0.85% (w/v) NaCl solution
10 ml. distilled water
0.6 ml. iodine solution (IIIb).

If the iodine colour of the test mixture is not blue-green, the serum contains too much amylase. Repeat the determination as follows: Rinse

10 µl. serum

into

0.5 ml. 0.85% (w/v) NaCl solution

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*) Exelo pipette (W. G. Flaig & Sons, Ltd., London, England) or Lang-Levy pipette (Microchemical Specialists Co., 1934 University Avenue, Berkeley 3, Calif., USA).

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contained in a tube at 37°C ("diluted serum") Warm

1.0 ml. buffered substrate solution (IV)

in a glass-stoppered test tube (15 cm. long, 1.25 cm. diameter) to 37°C. To this add

0.1 ml. of the diluted serum,

incubate for 15 min. at 37°C and proceed as described above.

Calculations

As described in the micromethod for a dilution of 1 : 10 (see p. 856). For more dilute serum apply the formula:

$$\frac{510}{E_S} \times (E_S - E_T) = \text{Street-Close units/100 ml. serum}$$

Stability of the Enzyme in the Serum Sample

Serum amylase is stable at room temperature for at least one week [19].

Normal Values

In 200 cases, with the two methods described here, the normal range of serum amylase was found to be between 6 and 33 Street-Close units/100 ml. serum [15, 20].

Inhibitors and Activators

According to *Vallee* et al. [21] certain α-amylases (*e.g.* from human saliva) contain at least 1 gram atom of calcium per mole of enzyme. Incubation with ethylene-diamine-tetra-acetate inhibits these amylases. Serum amylase is inhibited by citrate and activated by borate, but fluoride ions have no effect [22].

Appendix

Preparation of amylose

Stir 30 g. of potato starch into a cream with 150 ml. water and pour this into 1 500 ml. boiling 2% (w/v) NaCl solution. Stir the hot mixture mechanically until it is homogeneous and filter whilst hot, through muslin. To the cooled filtrate add 4.5 g. powdered thymol crystals (*p*-isopropyl-*m*-cresol) and stir 48 hours. Decant and discard the supernatant which contains amylopectin. Separate the precipitate of the amylose-thymol complex from the residual fluid by centrifuging, wash the precipitate six times with thymol-saturated water and four times with absolute ethanol. Spread out the residue on a glass plate and dry for 6 hours at 37°C with occasional grinding in a mortar. Yield: 3.6 g. amylose. Store the white powder at room temperature in a tightly-stoppered, brown bottle.

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19) *H. V. Street*, unpublished.
20) *H. V. Street* and *J. R. Close*, Nature [London] *179*, 164 [1957].
21) *B. L. Vallee, E. A. Stein, W. N. Sumerwell* and *E. H. Fischer*, J. biol. Chemistry *234*, 2901 [1959].
22) *H. V. Street*, Biochem. J. *76*, 10 [1960].