β-Glucuronidase

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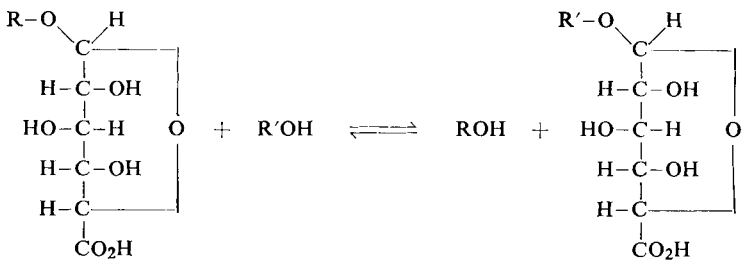
β-Glucuronidase is widely distributed in the tissues of mammals[1,2] and other animal species, bacteria[3], digestive juice of snails (*Helix pomatia*)[4], molluscs (*Patella vulgata*)[5], crop fluid of locusts[6], in plants[7] and fishes[8].

The enzyme catalyses the hydrolysis of β-glucuronides and also the transfer of glucuronyl radicals to acceptor alcohols[9,10]:



Substrates for the enzyme include the β-glucuronides of oestriol, oestradiol, testosterone, epiandrosterone, dehydroisoandrosterone, pregnanediol, pregnenolone, pregnanetriol, corticosteroids (*e.g* the tetrahydro derivatives of substances E and F), menthol borneol, orcinol, phloroglucinol, β-naphthol, phenolphthalein, bilirubin, chlorophenol and 8-hydroxyquinoline. α-Glucuronides or α- and β-glucosides are not hydrolysed.

Principle

The rate of hydrolysis of phenolphthalein glucuronide serves to assay the activity of β-glucuronidase. The phenolphthalein liberated is estimated by the red colour which it gives at alkaline pH[11,12]. Phenolphthalein glucuronide has hardly any absorption at the same pH.

Optimum Conditions for Measurements

The pH optimum for the enzymatic hydrolysis of β-glucuronides is below pH 6 (see Fig. 1)[1]. An exception is the enzyme from *E. coli.* which has a second optimum at pH *ca.* 7.5.

As a rule, the initial velocity of the hydrolysis is proportional to the substrate concentration, but at high substrate concentrations inhibition may occur. This inhibition does not occur in the presence of an alcohol acceptor.

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Activators and inhibitors: Deoxyribonucleic acid, albumin and a number of diamines of low molecular weight activate purified β-glucuronidase[14]. A substance occurs in rat liver which will also activate the impure enzyme[15]. A number of sugar acids and their lactones (saccharo-1 → 4-lactone, mucic and glucuronic acids) inhibit the enzyme. The existence of other inhibitors in tissue, serum and urine has been discovered.

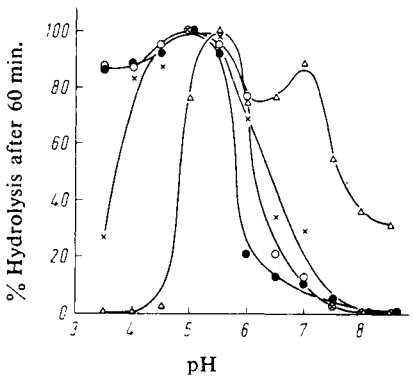

Fig. 1. Dependence on pH of the rate of hydrolysis of phenolphthalein glucuronide[13] by β-glucuronidase from
—○—○— *Helix pomatia*
—●—●— *Patella vulgata*
—×—×— Calf liver
—△—△— *Escherichia coli*

Reagents

1. Phenolphthalein glucuronide
   This substance is available commercially as the cinchonidine salt with 1.5 molecules of methyl alcohol of crystallization (*e.g.* Calbiochem). It is prepared biosynthetically[16] from the urine of rabbits which have been injected with phenolphthalein phosphate.
2. Hydrochloric acid, A. R., 2 N
3. Ethyl acetate
4. Sodium hydroxide, A. R., 0.1 N and 100% (w/v)
5. Sodium acetate·3 H_2O
6. Acetic acid, glacial
7. Trichloroacetic acid
8. Glycine
9. Sodium chloride
10. Phenolphthalein
11. Ethanol, absolute

Preparation of Solutions

I. Phenolphthalein glucuronide (0.01 M; pH 5.0):
Mix 0.837 g. cinchonidine salt with 20 ml. 2 N HCl, add 20 ml. ethyl acetate and stir until solution is complete. Transfer the mixture quantitatively to a separating funnel with several washings of ethyl acetate. Shake the separating funnel a 100 times. Decant the organic phase through an absorbent cotton wool plug in a powder funnel into a 500 ml. Erlenmeyer flask. Extract the aqueous phase six times with 20 ml. portions of ethyl

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acetate, decanting each time as described above. Evaporate the combined extracts *in vacuo* at room temperature or by drawing a current of clean dry air into the flask. The gum-like, colourless residue is the free phenolphthalein glucuronide. Add 20 ml. hot distilled water, adjust to pH 5.0 with 0.1 N NaOH (indicator paper) and transfer the solution to a 100 ml. volumetric flask with several washings of distilled water. Dilute with distilled water to the mark and mix thoroughly. If the solution is turbid, stir in a little activated charcoal and filter.

II. Acetate buffer (0.1 M; pH 4.5):
Dissolve 5.79 g. sodium acetate·3 H_2O in distilled water, add 3.25 ml. glacial acetic acid and make up to 1000 ml.

III. Trichloroacetic acid (5% w/v):
Dissolve 5 g. trichloroacetic acid in 100 ml. distilled water.

IV. Alkaline glycine solution (*ca.* 0.1 M):
Dissolve 16.3 g. glycine and 12.65 g. NaCl in distilled water, add 12.0 ml. conc. NaOH (100 g. NaOH/100 ml. distilled water) and make up to 1000 ml. Adjust the pH of this solution by addition of glycine or NaOH so that a mixture of 2.5 ml. of this solution, 1.0 ml. trichloroacetic acid solution (III), 1.0 ml. acetate buffer (solution II) and 1.5 ml. distilled water has a pH of between 10.2 and 10.45 *).

V. Phenolphthalein standard solution (1 mg./ml.):
Dissolve 100 mg. phenolphthalein in 100 ml. ethanol.

Stability of the solutions

All the solutions are stable. Store the substrate solution (I) in a refrigerator.

Procedure

Enzymatic reaction

Preliminary remarks: The method described here is suitable for studies on serum or plasma, providing that the sample is free from haemolysis and is not strongly coloured. Other samples necessitate modifications which are described in an appendix to this section.

Test tubes containing the substrate and buffer solution can be prepared in advance. They keep for several months at 4°C when stoppered.

Method: Wavelength: 540 mμ; measure against a blank (as for the experimental tube, but without sample).

Pipette into test tubes:

Experimental	*Control*
0.1 ml. substrate solution (I)	0.9 ml. buffer (solution II)
0.8 ml. buffer (solution II)	0.1 ml. sample
0.1 ml. sample.	

Mix by shaking gently, stopper the tubes and incubate for from 5 to 17 hours at 38°C (constant temperature water bath). Add to both tubes

2.5 ml. glycine solution (IV)
1.0 ml. trichloroacetic acid solution (III)

*) The intensity of the phenolphthalein colour depends on the pH and therefore periodic checks should be made to see whether the pH of the diluted mixture (solution IV + other components) is between 10.2 and 10.45.

and dilute with

distilled water to 6.0 ml.

Mix thoroughly and read the optical densities.

Details for assays on other types of sample

Haemolytic or coloured serum or plasma:

After the incubation add

1.0 ml. trichloroacetic acid solution (III),

centrifuge and wash the precipitate twice with

0.8 ml. portions of distilled water.

Add to the combined supernatants

2.5 ml. glycine solution (IV)
distilled water to 6.0 ml.

Mix thoroughly and read the optical densities.

Other body fluids:

Proceed as for serum, but prepare 3 experimental tubes: 0.1 ml. untreated sample; 0.1 ml. supernatant after centrifuging the sample; 0.1 ml. of a suspension of the sediment in distilled water (volume of the suspension = volume of the original sample).

Vaginal or cervical fluid:

a) Preparation of the sample: Mark small test tubes (1.3 cm. diameter, 10 cm. long) at 3 ml. and weigh. Weigh in the sample (not more than 100 mg.), add

1 ml. acetate buffer (solution II)

and homogenize the sample until a fine suspension is obtained. Lengthy homogenization can result in loss of activity due to the frictional heat. Rinse the homogenizer rod with a little acetate buffer (solution II). Make up the volume of the suspension

to 3 ml. with acetate buffer (solution II).

Use 0.1 ml. of this suspension for the assay.

b) Enzymatic reaction: Incubate overnight at 37° C. Immerse the tubes for 1 min. in a boiling water bath. Add

1.5 ml. distilled water

and centrifuge for 5 min. at *ca.* 2000 g.

Pipette off

2 ml. supernatant

and add

2.5 ml. glycine solution (IV)
1.0 ml. trichloroacetic acid solution (III)
distilled water to 6.0 ml.

Mix thoroughly and read the optical densities.

Tissue samples:

a) Preparation of the sample: Weigh out the tissue (50 to 100 mg.) into a glass homogenizer, mince with scissors, rinse the scissors and the upper part of the homogenizer with a little acetate buffer (solution II). Homogenize and continue to add buffer until a uniform suspension is obtained. Lengthy homogenization can lead to loss of activity due to frictional heat. Transfer the homogenate to a calibrated 15 ml. conical centrifuge tube and dilute to 10 ml. with acetate buffer (solution II)*). Shake the mixture thoroughly and use 0.1 ml. for the assay.

b) Enzymatic reaction: See "Vaginal and cervical fluids".

Standard curve:

Prepare a series of dilutions of the phenolphthalein standard solution (V), containing between 1 μg. phenolphthalein/ml. (dilute 0.1 ml. solution V to 100 ml. with ethanol) and 20 μg. phenolphthalein/ml. (dilute 0.1 ml. solution V to 5 ml. with ethanol). Pipette into test tubes:

1.0 ml. trichloroacetic acid solution (III)
1.0 ml. buffer (solution II)
2.5 ml. glycine solution (IV)
0.5 ml. distilled water
1.0 ml. dilute phenolphthalein standard solutions.

The pH of the mixtures must be between 10.2 and 10.45. Read the optical densities at 540 mμ against water and plot against the μg. phenolphthalein/tube (standard curve).

Calculations

Calculate the optical density difference $\Delta E = E_E - E_C$. (E_E = optical density of the experimental tube; E_C = optical density of the control tube). Obtain the amount of phenolphthalein corresponding to the ΔE from the standard curve.

The β-glucuronidase activity is given in *Fishman* units[12]. A *Fishman* unit is the amount of enzyme which liberates 1 μg. phenolphthalein from a 10^{-3} M solution of phenolphthalein glucuronide in 1 hour at pH 4.5 (0.1 M acetate buffer). The same definition also holds if the optimum pH of the enzyme is not 4.5, providing that the assay is carried out at the optimum pH. From this definition the following formulae for the calculation of the β-glucuronidase activity are derived:

Serum, plasma and other body fluids:

$$\frac{(\mu g.\ \text{phenolphthalein/tube}) \times 100}{(\text{period of incubation [hours]}) \times 0.1} = \text{units/100 ml. sample}$$

Vaginal or cervical fluid:

$$\frac{(\mu g.\ \text{phenolphthalein/tube}) \times 3 \times 2.5}{(\text{period of incubation [hours]} \times (\text{weight [g]}) \times 0.1 \times 2} = \text{units/g. sample}$$

Tissue samples:

$$\frac{(\mu g.\ \text{phenolphthalein/tube} \times 10 \times 2.5}{(\text{period of incubation [hours]}) \times (\text{weight [g.]}) \times 0.1 \times 2} = \text{units/g. sample}$$

*) The final concentration of the homogenate should be about 1% (w/v) because this ensures that all the β-glucuronidase is in solution[2].

Other Methods for the Determination of β-Glucuronidase

The following substrates can be used instead of phenolphthalein glucuronide:

8-Hydroxyquinoline glucuronide[6]. The 8-hydroxyquinoline liberated forms a dye with tetrazotized *o*-diansidine.

Umbelliferone glucuronide[17]. The umbelliferone liberated is fluorescent.

p-Chlorophenol glucuronide[18]. Alkaline solutions of the *p*-chlorophenol liberated absorb in the ultraviolet.

Purification of β-Glucuronidase

Purification procedures consist of the following steps:

Acid denaturation, ammonium sulphate fractionation and alcohol precipitation. β-Glucuronidase has been purified from spleen[19,20], liver[21], mouse kidney[22], preputial gland[23], digestive juice of *Helix pomatia*[24,25] and *E. coli*[26].

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