BBA 63236

pH-Dependent activation of β-D-glucuronidase by *N*-acetyl-β-D-glucosaminidase

Mammalian preparations of β-D-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) display a double pH optimum at pH 5.2 and 4.5 under appropriate conditions of assay. At low concentrations the enzyme is activated by compounds of high molecular weight such as bovine serum albumin and DNA. Albumin causes no appreciable change in the pH–activity curve, whereas on addition of DNA the optimum at pH 4.5 disappears[1,2].

In the course of preparation of highly purified β-D-glucuronidase from bovine arterial wall the following observations were made: in arterial tissue homogenates the specific activities of β-D-glucuronidase and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) were 0.44 and 14.0 milliunits, respectively (1 unit = 1 μmole of product per min per mg protein), *i.e.*, the ratio of their activities was 1:32. Despite an extensive purification procedure β-D-glucuronidase and *N*-acetyl-β-D-glucosaminidase activities could not be separated. Even after a 1200-fold purification of β-D-glucuronidase (with respect to the homogenate) the ratio of the specific activities of the two enzymes was still 1:5. Previous purification steps involved enzyme extraction with 1% sodium chloride, precipitation by ammonium sulfate (15–35%, w/v) and ethanol (50%, v/v; −10°), column chromatography on TEAE-cellulose (0.01 M phosphate buffer, pH 6.8, gradient elution 0–0.3 M NaCl) and on Sephadex G-100 and G-200 (as in Fig. 1). The elution pattern of the enzyme preparation in the last purification step (see Fig. 1) shows both activity peaks in practically the same position. This enzyme preparation, however, is stable only in solution. On lyophilization the β-D-glucuronidase activity disappears, while the *N*-acetyl-β-D-hexosaminidase activity remains constant. By this procedure a β-D-glucuronidase-free *N*-acetyl-β-D-hexosaminidase preparation was obtained and used in the activation experiments. The β-D-glucuronidase activity was assayed according to TALALAY, FISHMAN AND HUGGINS[3]. *N*-Acetyl-β-D-glucosaminidase activity was determined as described previously[4].

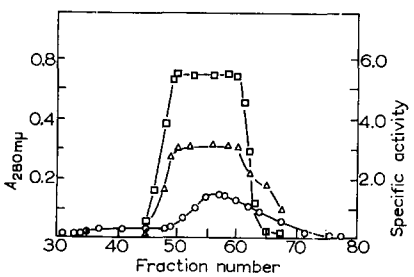

Fig. 1. Final-stage elution pattern from a Sephadex G-200 column (1.5 cm × 300 cm) of an arterial wall enzyme preparation containing β-D-glucuronidase and *N*-acetyl-β-D-glucosaminidase activities. 7 mg of the enzyme preparation was applied in 0.1 M citrate buffer (pH 5.2) containing 0.1 M NaCl. For previous purification steps see text. ○, absorbance at 280 mμ. Specific activity for β-D-glucuronidase (□) is given in units × 10^{-1}, and that for *N*-acetyl-β-D-glucosaminidase (△) in units.

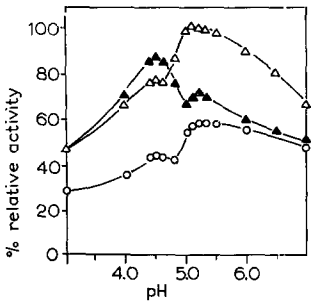

Fig. 2. pH-dependent activation of arterial wall β-D-glucuronidase by crystallized bovine serum albumin (△, 0.01%) and *N*-acetyl-β-D-glucosaminidase (▲, 0.01% protein containing 3.4 units enzyme). ○, non-activated β-D-glucuronidase containing 0.29 milliunit *N*-acetyl-β-D-glucosaminidase. Assay in 0.1 M acetate buffer with 0.005 M phenolphthalein glucuronide as substrate.

The pH–activity curve of purified β-D-glucuronidase in 0.1 M acetate buffer (Fig. 2) shows optima at pH 5.2 and 4.5, the ratio of β-D-glucuronidase activities at pH 5.2 and 4.5 being 1.3:1. Upon addition of crystallized bovine serum albumin (final concn. 0.01%) the known activation effect occurs; the ratio of the enzymatic activities at pH 5.2 and 4.5 remains constant. In contrast, the addition of β-D-glucuronidase-free *N*-acetyl-β-D-glucosaminidase, also in a final protein concentration of 0.01%, results in a predominant activation of β-D-glucuronidase at pH 4.5 and in a shift in the ratio of β-D-glucosaminidase activities at pH 5.2 and 4.5 from 1.3:1 to 0.82:1.

As shown in Table I the activation effect depends on the amount of *N*-acetyl-β-D-glucosaminidase added. At pH 4.5 the activity increases by 133%, at pH 5.2 only by 24%. Further addition of *N*-acetyl-β-D-glucosaminidase did not appreciably raise the glucuronidase activity. The activation phenomenon is not observed in citrate buffer.

TABLE I

pH-DEPENDENT ACTIVATION OF BOVINE ARTERIAL WALL β-D-GLUCURONIDASE BY INCREASING AMOUNTS OF BOVINE ARTERIAL WALL *N*-ACETYL-β-D-GLUCOSAMINIDASE

β-D-Glucuronidase concentration and conditions of assay as in Fig. 2. The added *N*-acetyl-β-D-glucosaminidase was free of β-D-glucuronidase.

N-Acetyl-β-D-glucosaminidase added		*μmoles phenolphthalein liberated per h per assay*	
μ protein	*milliunits*	*pH 5.2*	*pH 4.5*
0	0	0.090	0.055
8	27	0.103	0.085
16	54	0.106	0.110
24	82	0.110	0.118
40	135	0.112	0.128

The effect of bovine serum albumin and of *N*-acetyl-β-D-glucosaminidase on the rate of the glucuronidase–phenolphthalein glucuronide reaction at pH 5.2 and 4.5 is shown in Fig. 3. In acetate buffer the Michaelis constants (expressed in mM) for β-D-glucuronidase are 0.140 at pH 5.2 and 0.077 at pH 4.5 using phenolphthalein

glucuronide as substrate. When the β-D-glucuronidase was saturated with substrate, the highest rate of reaction was found at pH 5.2; at sub-optimal substrate concentration, however, the highest rate of hydrolysis was observed at pH 4.5.

The data presented reflect a close relationship between *N*-acetyl-β-D-glucosaminidase and β-D-glucuronidase. Another expression of this close relationship is their alternate action on oligosaccharides from hyaluronate and chondroitin 4-sulfate and their supposed localization in lysosomes within the cell. Activation of one enzyme by another one has been described for aldolase (EC 4.1.2.13) and glycerol-3-phosphate dehydrogenase (EC 1.2.1.12)[5], also acting in succession. Possible mechanisms explaining double pH optima of enzymes have been discussed by SCHWIMMER[6].

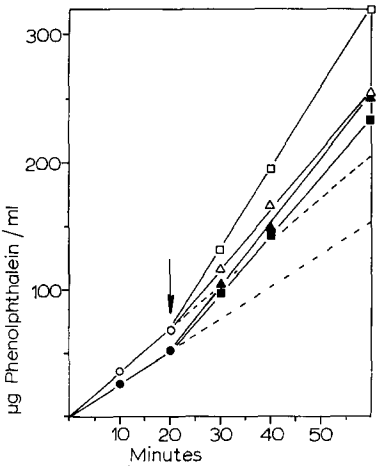

Fig. 3. Initial reaction velocity of β-D-glucuronidase (1.52 milliunits) at pH 5.2 (○) and pH 4.5 (●). After a preincubation period of 20 min bovine serum albumin was added in a final concentration of 0.01 % (□, ■) and 375 milliunits *N*-acetyl-β-D-glucosaminidase (△, ▲) giving a final protein concentration of 0.011 %. Ordinate: µg phenolphthalein liberated per ml assay.

Bovine arterial tissue (aorta) contains about 2 mg chondroitin 4-sulfate per g wet weight. Since the half-life time of arterial tissue chondroitin 4-sulfate is approx. 10 days, as ascertained by tracer studies[7,8], the rate of degradation will be 0.004 µmole/h (referred to the tetrasaccharide fragment as the molecular unit). Determination of β-D-glucuronidase activity with the tetrasaccharide as substrate[9] revealed an enzyme concentration of 0.015 unit/g arterial tissue wet weight. From this value it may be easily calculated that the β-D-glucuronidase concentration present in arterial tissue homogenates (referred to 1 g wet weight) is high enough to release 0.9 µmole glucuronic acid per h. It is thus evident that the physiological substrate concentration is only a small fraction of the saturation concentration for β-D-glucuronidase.

In the above calculation the assumption is made that the fraction of chondroitin sulfate which is turned over is present as an oligosaccharide susceptible to the β-D-glucuronidase. This assumption is based on the observation that glucuronidase-susceptible oligosaccharides result from the action of arterial wall hyaluronidase (EC 3.2.1.35) on chondroitin 4-sulfate[10].

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Inhibition of the trypsin-catalyzed activation of chymotrypsinogen by *N*-acetyl-L-3,5-dibromotyrosine

Chymotrypsinogen readily forms a stoichiometric equilibrium complex with reversible chymotrypsin (EC 3.4.4.5) inhibitors[1–4]. The great similarity of all the binding characteristics suggested that the binding sites are identical in chymotrypsinogen and chymotrypsin[1,4]. Thus, at least part of the active center of chymotrypsin is preformed in the zymogen molecule. In the trypsin (EC 3.4.4.4)-catalyzed activation of chymotrypsinogen an N-terminal isoleucine is formed which has been shown to control through its ionization the activity of chymotrypsin[5]. The localization of this isoleucine residue with respect to the binding site of chymotrypsinogen and hence the active center of chymotrypsin, is of primary importance in the elucidation of the mechanism of the control process. Studying the activation of the zymogen in the presence of inhibitors combining with its binding site is one possible tool for the determination of the relative position of the catalytically important isoleucine residue. If the latter is in close proximity to the binding site then the binding of an inhibitor to this site certainly would inhibit the activation of the zymogen. If, on the contrary, the isoleucine is widely separated from the binding site, then the binding of an inhibitor to the zymogen would only have a small secondary effect on its activation.

In order to decide between these two possibilities, we determined the influence of *N*-acetyl-L-3,5-dibromotyrosine on the trypsin-catalyzed activation of chymotrypsinogen. This particular inhibitor was chosen for the reason that its binding characteristics to the zymogen are accurately known[1]. The dissociation constant of the inhibitor–zymogen complex, as measured by equilibrium dialysis at pH 5 has a value of $0.83 \cdot 10^{-2}$ M at 5° and $0.985 \cdot 10^{-2}$ M at 20°.

If the activation process would occur at close proximity to the binding site and the zymogen–inhibitor complex would be totally unable to react with trypsin, then the activation reaction should be described by the following kinetic scheme:

$$Chtg \cdot I \underset{K_i}{\overset{+I}{\rightleftarrows}} Chtg + Try \overset{K_s}{\rightleftarrows} Try \cdot Chtg \xrightarrow{k_{cat}} Try + Cht$$

where *Chtg*, *I*, *Try* and *Cht* designate chymotrypsinogen, *N*-acetyl-L-3,5-dibromotyro-