Separation and Properties of β -Galactosidase, β -Glucosidase, β -Glucuronidase and *N*-Acetyl- β -glucosaminidase from Rat Kidney

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1. The activities of β -galactosidase, β -glucosidase, β -glucuronidase and *N*-acetyl- β -glucosaminidase from rat kidney have been compared when 4-methylumbelliferyl glycosides are used as substrates. 2. Separation by gel electrophoresis at pH 7.0 indicated slow- and fast-moving components of rat-kidney β -galactosidase. 3. The fast-moving component is also associated with the total β -glucosidase activity and inhibition experiments indicate that a single enzyme species is responsible for both activities. 4. DEAE-cellulose chromatography and filtration on Sephadex gels suggests that the β -glucosidase component is a small acidic molecule, of molecular weight approx. 40 000–50 000, with optimum pH 5.5–6.0 for β -galactosidase and β -glucosidase activities. 5. The major β -galactosidase component has low electrophoretic mobility, a calculated molecular weight of 80 000 and optimum pH 3.7.

In the rat the kidney is second only to epididymis as an active source of glycosidases (Conchie, Findlay & Levvy, 1959). Those identified so far are hydrolases with pH optima about 5 and similar enzymes have been shown by Sellinger, Beaufay, Jacques, Doyen & de Duve (1960) to be located in the lysosomes in liver.

The kidney enzymes also exhibit the phenomenon of latency when prepared as homogenates in iso-osmotic sucrose, but tissue fractionation experiments suggest that they do not all have the same distribution in the cell particles (Conchie & Hay, 1963), and a histochemical examination of rat organs by Rutenburg, Rutenburg, Monis, Teague & Seligman (1958) revealed that the tissue localization of β -galactosidase was not always the same as that for other lysosomal enzymes. An extensive survey of the enzyme content and characteristics of rat-kidney lysosomes was carried out by Shibko & Tappel (1965).

Although the β -glucosidase (β -D-glucoside glucosylhydrolase, EC 3.2.1.21) and β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) activities of almond emulsin and of pig intestines are attributed in each case to a common active site (Heyworth & Dahlqvist, 1962), Rutenburg *et al.* (1958) noted differences in the stability of these two enzymes to the fixing procedures used and concluded that they generally had a separate existence in mammalian tissues. The behaviour of rat-kidney extracts on DEAE-cellulose columns suggests that

a number of β -galactosidase components may be present (Furth & Robinson, 1965), and a similar study on pig kidney showed that, though it was possible to separate a β -galactosidase with no associated β -glucosidase activity, other components were present that could react with both substrates (Price & Robinson, 1966).

In the present paper some characteristics of rat-kidney β -galactosidase, β -glucosidase, β -glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31) and *N*-acetyl- β -glucosaminidase (β -2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucosylhydrolase, EC 3.2.1.30) have been examined and a particular study has been made of the relation of the β -galactosidase to the β -glucosidase component.

EXPERIMENTAL

Materials. 4-Methylumbelliferyl β -D-glucuronide was prepared biosynthetically as described by Mead, Smith & Williams (1955), 4-methylumbelliferyl β -D-glucoside by the method of Robinson (1956) and the corresponding β -D-galactoside and α -L-arabinoside by an analogous procedure (Robinson, 1964). 4-Methylumbelliferyl *N*-acetyl- β -glucosaminide was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Glucono-(1 $\rightarrow$ 5)-lactone and galactono-(1 $\rightarrow$ 4)-lactone were also from this source. Salts and other buffer constituents were A.R. grade (British Drug Houses Ltd., Poole, Dorset).

The standard preparations used as markers for chromatographic columns were as follows: horseradish peroxidase and cytochrome *c* (British Drug Houses Ltd.), *Escherichia*

coli alkaline phosphatase and rabbit-muscle aldolase (Sigma Chemical Co., St Louis, Mo., U.S.A.), *E. coli* β -galactosidase (autolysate of ML308 culture), Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) and sucrose (A.R.) (British Drug Houses Ltd.). The assumed molecular weights are those quoted by Andrews (1964, 1965).

Tissue preparations. Adult male Sprague-Dawley rats were killed by a blow on the head and the organs rapidly removed. Kidney homogenates (3%, w/v) were prepared in 0.01 M-sodium phosphate buffer, pH 6.0, by using a Potter-Elvehjem-type homogenizer fitted with a Teflon pestle. The 10^6 g-min. supernatant was used for column separations where completely soluble preparations were required. There was no substantial loss of enzyme in the sediment.

For gel electrophoresis the dilute enzyme samples obtained from column eluates were concentrated by dialysis under vacuum in 8/32in. Visking tubing at 4° against 0.04 M-sodium phosphate buffer, pH 7.0.

DEAE-cellulose chromatography. Whatman DEAE-cellulose powder DE50 (W. and R. Balston Ltd.) was prepared and used as described by Furth & Robinson (1965). A 2ml. sample of extract containing up to 50mg. of protein was applied. A linear NaCl gradient up to 0.3 M was used for elution at 4°, and 3ml. fractions were collected for protein and glycosidase assays. Columns prepared in the same way were also used with a step-wise chloride elution.

Gel filtration. Sephadex G-75 (200–300 mesh; Pharmacia Ltd.) was allowed to swell for 2 days in 0.01 M-sodium phosphate buffer, pH 7.6, containing 0.4 M-NaCl and 0.01 M-EDTA (Ada, 1963). The de-aerated suspension was packed under gravity in a 36cm. $\times$ 3cm. diam. column. Blue Dextran 2000 was used to indicate the void volume and sucrose to indicate the internal volume. The enzyme samples (containing up to 50mg. of protein) were applied as a solution in 2ml. of the same buffer and eluted at a flow rate of 10ml./hr. The eluate fractions (3ml.) were assayed for protein and glycosidases. Gel filtration through Sephadex G-200 was performed in a similar fashion.

Gel electrophoresis. Enzyme components were separated in starch gel at 4° in 0.04 M-phosphate buffer, pH 7.0 (Gammack, Huehns, Shooter & Gerald, 1960).

Proteins were detected with Amido Black stain, and enzyme activity by covering the cut face of the gel with filter paper and flooding with the appropriate substrate solution in buffer, as used for the quantitative assays. The gels were incubated for 5–15 min. at 37° to develop blue fluorescent bands, which were viewed under u.v. light (350 m μ). These can be intensified with aq. NH₃ (sp.gr. 0.88).

Assay of glycosidases. All the assays involved fluorimetric

estimation of 4-methylumbelliferone liberated from the appropriate glycoside under standard conditions. β -Glucuronidase was estimated as described by Mead *et al.* (1955), β -glucosidase and β -galactosidase by the methods of Price & Robinson (1966) and *N*-acetyl- β -glucosaminidase by that of Leaback & Walker (1961).

A suitably diluted sample of enzyme solution (1ml.) was added to 1ml. of the substrate in citrate-phosphate buffer (McIlvaine, 1921) or 0.1 M-acetate buffer, and incubated for 30 min. at 37°. The reaction was stopped and fluorescence developed by the addition of 3ml. of 0.5 M-glycine-NaOH buffer, pH 10.4. The appropriate substrate concentrations and pH optima for the individual enzymes are included in Table 1.

Fluorescence was measured in a Locarte LF.5 fluorimeter activating at 340–380 m μ and with the secondary-beam monochromator set at 440 m μ .

Michaelis constants. The apparent K_m values were calculated by the double-reciprocal plot method of Lineweaver & Burk (1934).

Assays of column effluents. Peroxidase was assayed at room temperature (18°) by adding 0.2 ml. of enzyme solution to 1 ml. of 4% (w/v) *o*-toluidine in acetic acid and 1 ml. of 3% (w/v) H₂O₂. After 2 min. the blue colour was read at 620 m μ on the Unicam SP.600 spectrophotometer. The alkaline-phosphatase assay of Garen & Levinthal (1960) was used, and aldolase was measured by the method of Sibley & Lehninger (1949). Cytochrome *c* was assayed by extinction measurements at 412 m μ , Blue Dextran 2000 at 625 m μ and protein at 280 m μ .

Sucrose was estimated with the Seliwanoff reagent. The sample (0.5 ml.) was mixed with 2 ml. of Seliwanoff reagent and heated for 10 min. in a boiling-water bath. The solution was cooled and diluted by the addition of 2.5 ml. of water and the colour measured at 420 m μ on the Unicam SP.600 spectrophotometer. Chloride was measured by the method of Sanderson (1952).

RESULTS

Comparative enzyme activities. Comparison of our results with those of previous workers is difficult since although many glycosidases have been assayed (Conchie *et al.* 1959) various synthetic substrates have been used. To minimize any variations in the effect of the aglycone on the lability of the glycosidic link the assays reported here were all performed with analogous substrates on portions of the same tissue samples. However, it is not

Table 1. *Characteristics and relative activities of β -glycosidases in rat kidney*

All assays were carried out in phosphate-citrate buffer (McIlvaine, 1921), except for β -glucuronidase, when 0.1 M-acetate buffer was used. Other details were as described in the text. The activities are expressed as μ moles of substrate hydrolysed/mg. of fresh tissue/hr. (no. of experiments in parentheses).

Enzyme	Final concn. of substrate (mM)	pH	Activity (mean $\pm$ S.D.)	K_m (mM)	V_{max}
<i>N</i> -Acetyl- β -glucosaminidase	0.264	4.5	191 $\pm$ 39 (9)	0.67	676
β -Galactosidase	0.50	3.7	70.8 $\pm$ 20.1 (9)	0.22	102
β -Glucosidase	0.50	5.5	32.5 $\pm$ 6.4 (9)	0.10	39
β -Glucuronidase	0.050	3.5	18.6 $\pm$ 2.1 (5)	0.02	21.4

possible in some cases to approach enzyme saturation because of the low solubility of the substrates, and the results in Table 1 are therefore also expressed as $V_{\max}$ values calculated from the observed activity at a known substrate concentration and the experimentally determined K_m values.

pH optima. It is well known that β -glucuronidase from other sources is profoundly affected by the buffer constituents and is inhibited by citrate ions (Mills, Paul & Smith, 1953; Woollen & Walker, 1965). The rat-kidney enzyme also shows differences in the pH optima and in absolute activity when assays in acetate buffer and in phosphate-citrate buffer are compared. Acetate is a competitive inhibitor for *N*-acetylglucosaminidase (Pugh, Leaback & Walker, 1957), and for the other enzymes examined in the present work phosphate-citrate mixtures seem to be the buffer of choice. Only β -glucosidase gave identical results in either buffer mixture (Fig. 1).

DEAE-cellulose chromatography. The gradient

elution pattern of a kidney homogenate (Fig. 2) confirms the results of Furth & Robinson (1965) that a major component of β -galactosidase is unretarded, followed by a number of fractions eluted by increasing chloride concentrations. The other three enzymes appeared to be less heterogeneous and had no unretarded components. A stepwise-elution technique (Fig. 3) gave partial separation of the enzymes and it is notable that β -glucosidase activity is only eluted after the bulk of the other enzymes has been displaced. The individual fractions were concentrated by vacuum dialysis and compared by gel electrophoresis with the patterns obtained from the crude homogenate.

Gel electrophoresis. Samples of kidney homogenate showed bands of activity moving slowly towards the anode for all the enzymes except β -glucosidase. The latter appeared as a sharp band of activity, migrating rapidly towards the anode and preceding the albumin band. A zone of β -galactosidase activity migrated similarly (Fig. 4).

Fig. 1. pH-dependence of rat-kidney glycosidase activity in acetate (open symbols) or citrate-phosphate (solid symbols) buffer. Activities are expressed as percentages of the maximum obtained for the particular enzyme under the most favourable conditions. (a) β -Glucosidase; (b) β -galactosidase; (c) *N*-acetyl- β -glucosaminidase; (d) β -glucuronidase.

Fig. 2. Chloride-gradient-elution patterns of rat-kidney β -glycosidases from DEAE-cellulose. The column (20 cm. $\times$ 1 cm.) was equilibrated with 0.01 M-phosphate buffer, pH 6.0, and eluted with an NaCl gradient (in the same buffer) up to 0.3 M. Each 3 ml. fraction was suitably diluted and assayed for the four enzymes by the method described in the text. (a) β -Glucuronidase; (b) β -glucosidase; (c) β -galactosidase; (d) *N*-acetyl- β -glucosaminidase; (e) chloride gradient. Activity is expressed in arbitrary units.

This could be due to a β -galactosidase component, which fortuitously had the same mobility as the β -glucosidase under these conditions, or alternatively it might represent the presence of a single enzyme having activity towards both substrates.

Fig. 3. Stepwise-chloride-elution patterns of rat-kidney β -glycosidases from DEAE-cellulose. Details of column and assay procedure were as described in Fig. 2 and the text. (a) β -Glucosidase; (b) β -galactosidase; (c) β -glucuronidase; (d) *N*-acetyl- β -glucosaminidase; (e) position and molarity of applied chloride steps.

To distinguish between these possibilities the gel patterns were developed with substrate solutions containing a high concentration (0.1%) of the appropriate glyconolactone inhibitor (Conchie & Levy, 1957). In the presence of glucono-(1 $\rightarrow$ 5)-lactone neither β -glucosidase nor β -galactosidase could be detected in the position of the fast-moving band, but fluorescence developed normally in the region of the slow-moving β -galactosidase bands. Galactono-(1 $\rightarrow$ 4)-lactone, on the other hand, inhibited the development of all the β -galactosidase and the β -glucosidase bands. It appears therefore that, though the major part of the kidney β -galactosidase is highly specific, a component is present that has the characteristics of almond-emulsin β -glucosidase (Horikoshi, 1942) in being non-specific for the configuration of C-4 of the sugar. This zone also showed hydrolytic activity towards 4-methylum-

belliferyl α -L-arabinoside and this was suppressed by the gluconolactone.

The β -galactosidase and β -glucosidase fractions from the stepwise elution on DEAE-cellulose were concentrated and examined in the same way. No electrophoretic differences could be seen in any of those β -galactosidase fractions that could be obtained before the elution of β -glucosidase, all of them having a similar slow mobility. The fast-moving β -galactosidase was confined to that eluate fraction which contained the β -glucosidase. This fraction is probably still heterogeneous and also contains a small amount of a slow-migrating β -galactosidase component.

Gel-filtration studies. When samples of the soluble fraction of kidney homogenates are applied to a

column of Sephadex G-75 and eluted as described the β -glucuronidase and *N*-acetyl- β -glucosaminidase activities emerge with the dextran marker and are thus of sufficiently high molecular weight to be unretarded. The β -galactosidase activity, when assayed at pH 3.7, appeared as an asymmetric peak with appreciable tailing into the later fractions where β -glucosidase occurs (Fig. 5). The leading edge of the peak gave samples that travelled slowly on electrophoresis, whereas the fast-moving β -galactosidase was confined to the later tubes that contained the β -glucosidase.

The deduction from these results that this latter fraction has a lower molecular size than the other components was confirmed in a more quantitative fashion by gel filtration through a Sephadex G-200 column. Commercial samples of enzymes whose molecular weights are known to close limits were used to standardize the column in the manner of Andrews (1965) and the positions of the elution peaks determined by their specific assay methods. A logarithmic plot of their molecular weights versus elution volumes was used and the unknown molecular weights were estimated from the positions of the peaks of glycosidase activity. The estimated molecular weight of rat-kidney β -glucuronidase is approx. 210 000. The molecular weight of the main kidney β -galactosidase component is about 80 000 and is thus very much smaller than that quoted for *E. coli* (510 000; Sund & Weber, 1963). The β -glucosidase activity, with its associated minor β -galactosidase component, occurs at a position that is consistent with a molecular weight of 40 000–50 000. *N*-Acetyl- β -glucosaminidase has an apparent molecular weight of 150 000–160 000.

Further characterization of β -glucosidase and β -galactosidase fractions. Estimations on crude homogenates of rat kidney gave optimum pH 3.7 for β -galactosidase and optimum pH 5.5 for β -glucosidase. However, if the two activities associated with the fast-moving band involve the same active site it might be thought that this fraction would exhibit the same optimum pH in both cases. When the eluate from a Sephadex G-75 column (Fig. 5) was assayed for β -galactosidase at pH 5.5 there was a depression of the peak value for the main component (peak I) but a clear rise in activity to a subsidiary peak in the β -glucosidase region (peak II). Further examination of the pH optima of the peak samples demonstrated (Fig. 6) that, though the bulk of the β -galactosidase activity (peak I) has a pH optimum of 3.7, that which is associated with the β -glucosidase (peak II) has a pH optimum of 6.0–6.5. The ratio of β -glucosidase to β -galactosidase activities in this fraction is about 3:1, and it comprises all the β -glucosidase and 15–20% of the total β -galactosidase activity of our

Fig. 4. Gel-electrophoresis patterns of rat-kidney β -glucosidase (a) and β -galactosidase (b). The separation was carried out at 4° in 0.04 M-phosphate buffer, pH 7.0, for 3 hr. and the bands were developed as described in the text. The fluorescence was recorded on Kodachrome II film, by using a Wratten 8 filter and illuminating with a Camag TL 900 u.v. lamp at 350 m μ .

Fig. 5. Separation of rat-kidney β -glucosidase and β -galactosidase on Sephadex G-75. The sample was applied in 2 ml. of 0.01 M-phosphate buffer, pH 7.6, to a column (36 cm. $\times$ 3 cm.) and eluted in the same buffer at a flow rate of 10 ml./hr. Other details were as described in the text. ○, β -Glucosidase assayed at pH 5.5; □, β -galactosidase assayed at pH 5.5; ■, β -galactosidase assayed at pH 3.7.

Fig. 6. pH-activity curves for peaks I and II from the Sephadex G-75 column separation (Fig. 5). Activities were measured in citrate-phosphate buffer mixtures and plotted as percentages of the activity at the optimum pH. Other details were as described in the text. O, β -Glucosidase; $\square$, low-molecular-weight β -galactosidase; $\blacksquare$, major β -galactosidase component.

preparations. A quantitative examination of the inhibitory effect of glucono- and galactono-lactones on these two peaks clearly demonstrates that both activities associated with peak II are strongly inhibited by either lactone at low concentrations, whereas the peak I β -galactosidase is only inhibited strongly by the galactonolactone (Fig. 7).

DISCUSSION

By performing the present assays on substrates carrying the same aglycone it was hoped to minimize differences in its influence on the lability of the glycosidic bond, so that the results might represent, as far as experiments with synthetic substrates can, the relative activities one might expect to find towards potential natural substrates such as kidney globoside and mucoproteins.

If this approach is valid then it becomes apparent that β -glucosidase activity, being present in the lowest amount of those enzymes studied here, might be the rate-limiting factor in the breakdown of mucoids; it is noteworthy that human spleen samples from cases of Gaucher's disease have subnormal levels of β -glucosidase activity (Patrick, 1965). The metabolic block causing the accumulation of gluco-cerebroside in this disease is attributed by Brady, Kanfer & Shapiro (1965) to a deficiency in the enzyme that cleaves the glucosidic link, rather than excessive glucoside synthesis. Similar accumu-

Fig. 7. Inhibiting effect of glucono-(1 $\rightarrow$ 5)-lactone (solid symbols) and galactono-(1 $\rightarrow$ 4)-lactone (open symbols) on the enzyme components of peaks I and II (Fig. 5). $\square$ and $\blacksquare$, β -Galactosidase; O and $\bullet$, β -glucosidase. (a) Effect on peak I; (b) effect on peak II. Activities are expressed as percentages of the control (uninhibited) value for a given enzyme component. Assay details were described in the text.

lations of glycolipid occur in the Gaucher and Fabry kidney (Sweeley & Kionsky, 1963), but there has been no investigation of altered enzyme activities in this organ.

The elution patterns obtained from DEAE-cellulose columns should be interpreted with caution in the absence of further evidence for the separate identity of multiple forms. For example, Jermyn (1962) has claimed that the presence of complex-forming polysaccharides may influence the behaviour of an otherwise homogeneous β -glucosidase preparation from *Stachybotrys atra*, so

as to give multiple peaks that suggest isoenzyme forms. Very high concentrations of carbohydrate have been reported in purified calf-intestinal β -galactosidase (Wallenfels & Fischer, 1960), but the enzyme from various strains of *E. coli* had no non-protein components (Wallenfels & Malhotra, 1960; Craven, Steers & Anfinsen, 1965). Butterworth & Moss (1966) suggested that the structurally different alkaline phosphatases of human kidney possibly arose from varying amounts of sialic acid bound to a single enzyme species, since they were able to reduce the electrophoretic mobilities of each of the various fractions to that of the slowest by treatment with neuraminidase. In the present case, however, there is clear evidence of distinct differences between the main β -galactosidase components. Not only does the electrophoretically fast band of β -galactosidase behave differently from the slow-moving β -galactosidase on DEAE-cellulose, but it also appears to have approximately half the molecular weight and a broader substrate specificity. Attempts to decrease the mobility of the β -glucosidase by prolonged incubation with neuraminidase have so far met with no success, nor are the slow-moving β -galactosidase components depolymerized into the fast-moving material by this treatment. If such an interconversion of the forms could take place it would involve considerable changes in specificity, for though the larger and slow-moving β -galactosidase appears to be highly specific the rapidly migrating component is essentially a β -glucosidase with associated β -galactosidase and α -arabinosidase activity, the ratio of β -glucosidase to β -galactosidase activities being about 3:1. There may, however, be further heterogeneity within these fractions. The pH-activity curves of the fast-band β -glucosidase and β -galactosidase cannot be superimposed and there is a difference in the pH optima. The curves suggest that there may in fact be two components in this fraction, both able to hydrolyse either substrate, but with different relative affinities. The inhibition studies to some extent support this contention, for although both inhibitors are effective the two enzyme activities are not equally inhibited at any given inhibitor concentration.

When, after gel electrophoresis, the slow-moving component is examined before the liberated aglycone can diffuse appreciably, it is seen to comprise two closely related bands (Fig. 4). No attempt has been made so far to examine these separately. As expected, this material shows a considerable sensitivity to galactonolactone inhibitor whereas gluconolactone has much less effect. There is, however, an initial 25% inhibition by 0.3 mM-gluconolactone that thereafter is not increased by higher concentrations and is as yet unexplained.

Multiple forms of mammalian β -galactosidase

have been reported from other sources. Chytil (1965) found two components in ox liver by electrophoresis at pH 4.0. The slower-moving component had optimum pH 4.5 and an estimated molecular weight of 85 000, whereas the fast-moving component (optimum pH 6.0) was half this size and hydrolysed β -glucosides more rapidly than β -galactosides. The similarity between these results and our findings on the kidney enzymes of rat is striking. Doell & Kretchmer (1962), working with rat-intestinal β -galactosidases, separated a particulate enzyme with optimum pH 6.0 from a soluble form with an acid pH optimum, and the rat-kidney enzymes described in the present work may also originate in different cell fractions.

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