Purification & Properties of Hetero-ß-galactosidases from Monkey Liver & Intestine

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Hetero-β-galactosidases have been purified from soluble fractions of monkey liver and intestine. The enzymes from both sources have a broad pH activity profile with α-naphthyl-β-D-galactoside as a substrate. The maximum activity is at pH 5.6 for the liver enzyme and between pH 6.5 and 7.0 for the intestinal enzyme. Both liver and intestinal enzymes act on only synthetic hetero-β-glycosides and have no action on lactose, cellobiose and phlorizin. The available evidence suggests that the β-galactosidases and β-glucosidases are hydrolysed by the same enzyme from both sources. There is no evidence of heterogeneity in both cases. Heavy metal ions like Hg²⁺, Ag⁺ and Cu⁺ inhibit both liver and intestinal enzyme, suggesting that probably both are sulphydryl enzymes.

The hetero-β-galactosidases hydrolyse primarily the synthetic galactosides and, in general, have very little or no action on lactose. The presence of soluble hetero-β-galactosidase has been demonstrated in many mammalian tissues like pig kidney, monkey intestine, rat and beef liver, rat spleen and kidney, ram testis, human liver, pig intestine, rat and human small intestine and bovine liver plasma membrane. In the liver, hetero-β-galactosidase is most probably a lysosomal enzyme. Purified preparations of lactase from monkey intestine or kidney have very little activity towards hetero-β-galactosidase.

The present work deals with the purification and characterization of the soluble hetero-β-galactosidases from monkey liver and small intestine. Evidence is presented to indicate that at least in the case of monkey liver and intestine the hetero-β-galactosidase and hetero-β-glucosidase activities are exerted by the same enzyme. Some of the properties of a less purified enzyme from monkey intestine have been studied earlier, and in this paper the properties of an enzyme with higher purity have been compared with those of a highly purified preparation from monkey liver. A preliminary report has already appeared.

Materials and Methods

Chemicals — The following chemicals were obtained commercially as indicated: β-nitrophenyl β-D-glucoside, β-nitrophenyl a-D-galactoside, α-nitrophenyl β-D-galactoside, phenyl β-D-galactoside and phenyl β-D-glucoside from National Chemical Laboratory, Poona; salicin, β-methyl 6-glucose, glucose oxidase, o-dianisidine, peroxidase, glucono-δ-lactone, galacto-δ-lactone, and bovine serum albumin from Sigma Chemical Co. Triton X-100 was a gift from Rohm & Haas Co. 3,5-Dinitrosalicylate was obtained from E. Merck. All other chemicals were of analytical or the best available grade.

Preparation of homogenates — Monkeys (Macaca mulatta) were anesthetized with Nembutal before being killed. The whole liver and the entire length of the intestine were removed. After cleansing the tissues, they were homogenized as described earlier. The tissues were homogenized with 0.25M sucrose containing 1 mM EDTA and a 50% homogenate was prepared in the case of liver and a 20% homogenate with intestine. Particle-free supernatant fractions were obtained by centrifuging the crude homogenates at 105,000 × g for 60 min using a preparative ultracentrifuge (Spinco, model L).

Assay of enzyme activities — The hetero-β-galactosidase and the hetero-β-glucosidase activities using synthetic substrates were determined as described earlier. In the case of liver enzyme sodium citrate-phosphate buffer (pH 5.6, 100 μmol) was used and in the case of intestinal enzyme Na-citrate-phosphate buffer (pH 7.0, 100 μmol) was used in the assay mixture.

Enzyme units — One unit of the enzyme activity is defined as the amount of enzyme required to hydrolyse 1 μmole of the substrate/min at 37°C. The specific activity is expressed as units/mg protein.

Polyacrylamide gel electrophoresis — Gel electrophoresis was carried out by the method of Davis using a Buchler instrument with 0.03M barbital buffer (pH 8.0) for 8 hr. The sample (1.0 ml of the DEAE-pooled fraction mixed with 1 ml of 40% sucrose) was placed on the spacer gel (2 ml) and electrophoresis was carried out using 8 mA/tube. After the run, the gels were washed out and cut into 1 cm segments from the cathodic end. The gels were homogenized in a Potter-Elvehjem homogenizer using 0.05M Na-phosphate buffer (pH 6.0) and centrifuged. The supernatant fraction from each segment was used for the assay. There was no enzymatic activity in the spacer gel.

Protein estimation — Protein was determined by the procedure of Lowry et al. using crystalline bovine serum albumin as standard.

Purification of Hetero-β-galactosidase

All operations were carried out at 0-5°C unless otherwise stated. The purification procedure employed for the liver enzyme is given below. The procedure was almost the same for the intestinal enzyme, except where indicated.

Step 1 — To the particle-free supernatant fraction from monkey liver (540 ml) 122 g of solid ammonium sulphate were added to give a saturation of 40%.
The precipitate obtained by centrifugation was discarded and to the supernatant fraction 91 g of ammonium sulphate were added to raise the saturation to 70% and then centrifuged. The precipitate containing the enzyme activity was suspended in 1 mM Na-phosphate buffer (pH 6-8) and was dialysed against the same buffer overnight.

Step 2 — An aliquot (135 ml) of the fraction obtained by the above step was adjusted to pH 5-0 by careful addition of 0.1 N acetic acid and an inactive precipitate was removed by centrifugation. The pH of the supernatant fraction was adjusted to 7-0.

Step 3 — To 140 ml of the fraction from step 2 (5.46 g protein) buffer washed bentonite (10.92 g) was added with vigorous stirring and allowed to stand for 15 min. It was then centrifuged at 10000 x g for 20 min. The pellet was discarded.

Step 4 — The supernatant fraction (100 ml) from step 3 was concentrated by the addition of 51-6 g of ammonium sulphate and the precipitate obtained by centrifugation was suspended in 1 mM Na-phosphate buffer (pH 6-8) and dialysed overnight. An aliquot (5 ml) of the concentrated sample was applied on a Sephadex G-200 column (2.5 cm x 25 cm; bed volume 100 ml). The column was washed with 50 mM Na-phosphate buffer (pH 6-8) and 2 ml fractions were collected. The flow rate was 5 ml/hr. The active fractions (fraction Nos. 27-38) were pooled and dialysed overnight against 1 mM Na-phosphate buffer (pH 6-8).

Step 5 — The dialysed fraction from the Sephadex G-200 column was applied on DEAE-Sephadex column (1 cm x 6 cm). The column was preequilibrated with 10 mM Na-phosphate buffer (pH 6-8). For elution a linear gradient (Varigrad device, Technicon) of sodium chloride (0-0-2 M) in 10 mM Na-phosphate buffer (pH 6-8) was used. The flow rate was 8 ml/hr. Sixty fractions of 2 ml size were collected and the enzymatically active fractions were pooled (Fig. 1).

In the case of intestinal tissue, Sephadex G-200 gel filtration after bentonite treatment was omitted and instead alumina C₄ gel fractionation was employed. When a ratio of 1 mg protein to 2.5 mg of gel was used, the intestinal enzyme was not adsorbed by the gel and was present in the supernatant fraction obtained after centrifuging. This fraction was then subjected to the DEAE-Sephadex chromatography as described for the liver enzyme.

By the above procedures, the liver enzyme was purified about 540-fold with a recovery of 10% and the intestinal enzyme 110-fold with a recovery of 4%. A summary of the purification procedure is given in Table 1.

**Results**

**pH optima** — With the crude enzyme from the liver using o-nitrophenyl β-D-galactoside as substrate, there was maximal activity in the range of pH 4-7, showing a hump each at pH 4-2 and pH 7-0 and with a peak at pH 5-6 with a slightly enhanced activity. This unusual pH-activity profile persisted even after purification (Fig. 2). Almost a similar profile was obtained when the substrate was o-nitrophenyl β-D-galactoside. However, when the substrate was β-nitrophenyl β-D-galactoside, the pH-activity profile changed to a smooth broad curve, with a very slight hump at pH 7-4 and with abolition of the hump at pH 4-2. The peak activity with this substrate was also at pH 5-6 (Fig. 3). With the intestinal enzyme using o-nitrophenyl β-D-galactoside as substrate, there was maximal activity in the pH range 4-3-7-5, showing a hump from pH 4-2 to 6-0, followed by a minor enhanced peak in the pH range 6-5-7-5 (Fig. 4).

The kinetic studies were carried out using o-nitrophenyl β-D-galactoside as substrate at pH 5-6 for the liver enzyme and at pH 7-0 for the intestinal enzyme.

**Substrate specificity and Kₘ values** — The purified enzyme preparations from both liver and intestine hydrolysed various synthetic hetero-β-galactosides and hetero-β-glycosides. The Kₘ values for the different substrates were of the same order for the enzymes from both sources. The Kₘ and Vₘₐₓ values are given in Table 2. There was no activity with lactose, cellobiose and phosphoribon as substrates in both cases.
Ion-exchange chromatography — The liver enzyme when fractionated on DEAE-Sephadex emerged as a single peak irrespective of whether the activity in the fractions was measured at pH 4.2, 5.6 or 7.4. There was thus no evidence for heterogeneity. The intestinal enzyme also emerged as a single peak very close to the position of the liver enzyme (Fig. 1).

Mixed substrate incubation studies — Mixed substrate incubation studies for both liver and intestinal enzymes with phenyl β-D-glucoside and various concentrations of phenyl β-D-galactoside showed mutual competition (Table 3). A similar competition was

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**Table 2 — Km and Vmax Values**

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Km (mM)</th>
<th>Vmax (μmoles of substrate hydrolysed/min mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Intestine</td>
</tr>
<tr>
<td>o-Nitrophenyl β-D-galactoside</td>
<td>8.6</td>
<td>8.9</td>
</tr>
<tr>
<td>β-Nitrophenyl β-D-galactoside</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Phenyl β-D-galactoside</td>
<td>10.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Phenyl β-D-glucoside</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Salolin</td>
<td>3.0</td>
<td>2.5</td>
</tr>
<tr>
<td>β-Methyl D-glucoside</td>
<td>8.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*α-lactase, cellobiose and pullulan were not active as substrates.

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**Table 3 — Mixed Substrate Incubation Studies on Purified Hetero-β-Galactosidase from Monkey Liver and Intestine**

(Reaction mixtures contained substrates separately or in a mixture at the indicated final concentrations. Other conditions are as mentioned in Materials and Methods. Purified enzyme was used for both the tissues. Figures in parentheses are values assuming no inhibition)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. (mM)</th>
<th>Phenol formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Intestine</td>
</tr>
<tr>
<td>Phenyl β-D-galactoside</td>
<td>2.5</td>
<td>38.5</td>
</tr>
<tr>
<td>Phenyl β-D-glucoside</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Phenyl β-D-glucoside + β-D-galactoside</td>
<td>3.0</td>
<td>22.0 (46)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>15.0 (22)</td>
</tr>
</tbody>
</table>
also observed with the p-nitrophenyl substrates (not shown in Table 3).

Inhibitors — EDTA had no effect on either the liver or the intestinal enzymes. Cu²⁺, Ag⁺ and Hg²⁺ at a concentration of 1 mM inhibited completely both the liver and the intestinal enzymes. Mg²⁺ at a concentration of 1 mM had no effect on both intestinal and liver enzymes. Glucose-6-lactone inhibited the enzyme activity more than did galactono-γ-lactone in both instances. Tris (0.1 M) inhibited the intestinal enzyme completely, whereas the liver enzyme was inhibited about 70%. All these inhibitors were studied using o-nitrophenyl β-D-galactoside as the substrate (Table 4).

**Table 4** — Effect of Metal Ions, EDTA, Lactones and Tris on Purified Hetero-β-Galactosidases

<table>
<thead>
<tr>
<th>Additions</th>
<th>% Activity</th>
<th>Liver</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ag⁺</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>98</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Galactono-lactone</td>
<td>80</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Glucose-lactone</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>59</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

Disc electrophoresis of the liver enzymes — Disc electrophoresis using polyacrylamide gel was carried out as described under Materials and Methods. Both the hetero-β-galactosidase and hetero-β-glucosidase activities were localized in a major protein band 3 cm from the cathode end. The recovery of enzyme from the gel slices after electrophoresis was only 16-18% of the activity applied and was the same for both β-galactosidase and β-glucosidase activities (Fig. 5).

Discussion

The results presented in this paper show that the supernatant hetero-β-galactosidase from monkey liver and intestine resemble each other in several properties. Using o-nitrophenyl β-D-galactoside as substrate for determining pH-activity profile, it was observed that the purified enzymes from both sources showed maximal activity over a wider range of pH. In the case of the liver enzyme, there were two peaks at pH values 4.2 and 7.0 and a peak at pH 5.6. This pH activity profile persisted even with the purified liver enzyme. It was first thought that this profile may be due to the presence of isoenzymes with different pH optima. Fractionation of the enzyme on DEAE-Sephadex columns and assaying for enzyme activity at the three different pH optimum values did not reveal any heterogeneity. When the fractions were assayed at the pH values of 4.2, 5.6 and 7.0, a single peak of activity was obtained. However, when the pH-activity profile was determined using phenyl β-D-galactoside as a substrate it was seen that the humps observed with o-nitrophenyl β-D-galactoside as substrate at pH values 4.2 and 7.0 could substantially be eliminated. The peak activity, however, remained at pH 5.6. So it is possible that the aglycon moiety of the substrate rather than the existence of isoenzymes is responsible for the type of pH-activity profile obtained. Chyiù has reported that with partially purified beef liver enzyme using o-nitrophenyl β-D-galactoside as substrate, there were three peaks in the pH-activity profile. Gel filtration studies demonstrated the presence of two enzymes, one having a pH optimum at 4 and the other at 6. However, gel filtration studies on monkey liver enzyme did not show any heterogeneity. In the case of rat liver, the enzyme is reported to have a single sharp peak at pH 5.5 with no activity beyond pH 4.5, and in human liver it is optimally active at pH 3.5 and is unstable below pH 4.5.

Both the monkey liver and the intestinal enzymes act on a variety of synthetic β-glycosides, but they have no action on cellobiose, phlorizin and lactose. The β-glucosidase activity was about 50% of the β-galactosidase activity in the case of liver, and was 40-75% in the case of intestinal enzyme depending on the hetero-β-glucosidase used. In rat liver homogenates, β-glucosidase activity has been shown to be absent.

Mixed substrate incubation studies and the disc electrophoresis data would strongly suggest that at least in monkey liver and intestine the synthetic hetero-β-galactosides and hetero-β-glucosidases are hydrolyzed by the same enzyme. In rat brain using deoxycholate, a partial separation of β-glucosidase and β-galactosidase has been achieved. In pig kidney, a supernatant β-glucosidase having β-galactosidase activity has been reported.
Heavy metal ions like Cu++, Ag⁺, and Hg²⁺ inhibit both monkey liver and intestinal enzymes, suggesting that these are sulphhydryl enzymes. Mercaptol like PHMB have inhibitory effect on only hetero-
β-galactosidase but not on lactase. EDTA has no effect on both liver and intestinal enzymes, thus suggesting that no metal ions are needed for activity. Mg²⁺ ions have no effect and in this regard the mammalian hetero-β-galactosidase seem to be different from the β-galactosidase of E. coli. Galactono-γ-lactone and glucono-δ-lactone inhibit both liver and intestinal enzymes, respectively, to the same extent, suggesting that both β-galactosidase and 8-glucosidase activities are exhibited by the same enzyme. This inhibits the enzyme from both sources and the difference in the extent of inhibition is different probably because of the differences in the pH at which the inhibition is measured. In the rat intestine, a hetero-β-galactosidase with an acid optimum pH, exhibiting latent activity and also acting on lactose, was demonstrated. This acid enzyme was lysozyme, and upon isoelectric focusing it was separated into 4 different forms, thus suggesting that the lysozymic acid hetero-β-galacto-
sidase is a high molecular aggregate.

In the human intestine a soluble hetero-β-galacto-
sidase with an optimum pH between 5 and 6 has been reported. The particulate acid hetero-
β-galactosidase was also present and could be sepa-
rated into three fractions on gel filtration; these frac-
tions are believed to be the disulfated part of the same protein. Gray and Santiago using gradient
density centrifugation, isolated two hetero-β-galacto-
sidase, one with a molecular weight of 60,000 and an optimum pH of 6 and the other with an opti-
mum pH of 4.5 existing as two species of molecular weight 156,000 and 660,000. The pH 6 enzyme hydrolysed only synthetic substrates, whereas the acid enzyme hydrolysed both lactose and synthetic substrates. The physiological significance of this enzyme is not clear at present. It has been reported that in the intestinal tissue of patients who have a primary deficiency of lactase, normal levels of hetero-β-galacto-
sidase are present. Whether these enzymes have any role in the degradation of β-linked galactose from glycoproteins or other higher molecular weight compounds needs investigation.

Acknowledgement

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