Characterization of Two Hetero-β-Galactosidases from Monkey Small Intestine

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Two hetero-β-galactosidase have been demonstrated in monkey small intestine and have been differentiated by their cellular location and kinetic properties. The pH optima of the partially purified "particulate" and "supernatant" β-galactosidases were found to be 4.5 and 7.0 respectively. The particulate enzyme also catalyzes the hydrolysis of lactose while the supernatant enzyme has no demonstrable activity toward lactose and this activity is not due to contamination by the major intestinal lactase. Both of the enzymes hydrolyze β-glucosides in addition to a variety of β-galactosides. Heavy metal ions and p-hydroxymercuribenzoate are inhibitory and the inhibition by the latter could be reversed by β-mercaptoethanol. No metal ion requirement could be shown.

It has been shown by earlier workers that lactose and synthetic β-galactosides are hydrolyzed by different enzymes in the mammalian intestine. Dolle and Kretzmer (1) from their studies on subcellular fractionation found that in the rabbit and rat intestines there are two β-galactosidases (β-galactoside galactohydrolase EC 3.2.1.23) showing different specificities towards lactose and o-nitrophenyl β-galactoside. Koldovsky et al. (2) and Dahlqvist and Asp (3) found different pH optima for the hydrolysis of lactose and phenyl and phenyl nitrophenyl β-galactoside. These data are difficult to evaluate since different substrates were employed. Swaminathan and Radhakrishnan (4) showed that the monkey intestinal lactase was different from the enzyme(s) hydrolyzing o-nitrophenyl β-galactoside (hetero-β-galactosidase) as distinguished by heat inactivation, distribution in the cell and along the intestine, as well as inhibition studies. Chromatography on DEAE-Sephadex gave two clearly separated peaks of lactase activity (4) but differing kinetically only to a minor degree. Lactase was the most active

substrate with cellobiose showing about 25% of the lactase activity, the ratio remaining constant in the various fractions. The minor (6%) associated hetero-β-galactosidase activity [(o-nitrophenyl β-galactoside and β-methyl glucoside) in the lactase preparations was probably exerted by the lactase itself (see Ref. 15). In addition to the lactase mentioned above, further investigations revealed the presence of two hetero-β-galactosidases distinguished by their cellular location, pH optima, and kinetic properties. The present paper deals with the nature of these two hetero-β-galactosidases from monkey intestine.

MATERIALS AND METHODS

Chemicals. p-Nitrophenyl-β-o-glucoside, p-nitrophenyl-β-o-galactoside, o-nitrophenyl-β-o-lactoside, phenyl-β-o-galactoside, phenyl-β-o-glucoside, 6-β-methyl-β-o-galactoside, 6-branched 2-naphthyl-β-o-galactoside, 6-branched 2-naphthyl-β-o-glucoside, zafelin (saligenin-β-o-glucoside), β-methyl glucoside, glucose oxidase, glucono-δ-lactone, genatase-γ-lactone, peroxidase, p-dimethylaminozinc and 6-bromo-2-naphthol were purchased from Sigma Chemical Company, U.S.A. Lactose and glucose were from B.D.H., Triton-X-100 was a gift from Rohm and Haas; 3,5-dinitrosalicylate was from E. Merck; diaco-

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naphthothenil blue B was obtained from Dajaol Labs., U.S.A.

METHODS

Assay of hetero-β-galactosidase activity. β-Galactosidase activity was determined by different methods depending on the substrate employed. The actual details of the incubation and the conditions of assay are given below. The assay conditions were identical for both the particulate and supernatant enzymes except for the pH of the incubation mixture. The pH was 7.0 (phosphate buffer) for the supernatant enzyme and 4.5 (acetate buffer) for the particulate enzyme.

The liberation of nitrophenol from 4-nitrophenyl-β-D-galactoside and other nitrophenyl-substituted substrates was measured according to the method of Seidman and Link (5) modified by Hubscher et al. (6). The reaction mixture in a total volume of 0.5 ml contained 0.5 mmoles of buffer, 5 amoles of nitrophenyl-β-D-galactoside or β-glucoside, and suitably diluted enzyme. After incubation at 37° for 20 min 2.5 ml of 0.4 M glycine-NaOH buffer, pH 10.6, was added to stop the reaction. After centrifugation the color of the supernatant fluid was measured in a Klett colorimeter using filter No. 42.

The liberation of phenol from phenyl-β-D-galactoside and β-glucoside was estimated with Polin-Ciecalante reagent as employed by Firth and Robinson (7) with slight modifications. The reaction mixture contained 5 amoles of phenyl-β-D-glucoside or β-D-galactoside, 50 amoles of lithium malonate buffer, pH 0.5 (use of phosphate buffer resulted in turbidity) and enzyme in a total volume of 0.5 ml. After 20 min incubation at 37°, 1.5 ml of water was added to all the tubes followed by 1 ml of Polin-Ciecalante reagent. The solution was centrifuged and 2 ml of 20% sodium carbonate was added to the supernatant fluid. After 20 min at 37° the blue color was read in a Klett colorimeter using filter No. 66.

The 2-naphthol liberated from the 6- bromo-2-naphthyl-β-D-galactoside and β-glucoside was estimated by the procedure of Dahlyquist et al. (8). The reaction mixture in a total volume of 5 ml contained 1.5 mg of 6-bromo-2-naphthyl-β-D-galactoside or β-glucoside, 500 amoles of buffer and enzyme. After 60-min incubation at 37° the reaction was stopped by placing in a boiling waterbath for 2 min. The rest of the procedure was the same as described by Dahlyquist et al. (8). The color was read in a Klett colorimeter using filter No. 54. A standard curve was obtained using 6-bromo-2-naphthol. In some experiments the reaction mixture was reduced to 1 ml and the additions were correspondingly scaled down.

Hydrolisis of lactose and β-methyl glucoside was measured using the Tris-glucone oxidase-peroxidase reagent of Dahlquist (9). Lactose or β-methyl glucoside (10 amoles) in 0.1 ml of 0.1 M buffer was incubated with enzyme in a total volume of 0.2 ml for 60 min at 37°. The reaction was stopped by placing the tubes in a boiling waterbath for 2 min. 0.3 ml of water added to all the tubes followed by 3 ml of Tris-glucone oxidase-peroxidase reagent and incubated at 37° for 60 min. The color was read in a Klett colorimeter using filter No. 62.

Hydrolisis of salicin was measured by following the formation of glucose by using 3,5-dinitrosalicylate reagent of Sommer (10). A suitable aliquot of the enzyme was incubated with 50 amoles of salicin and 100 amoles of buffer for 30 min at 37° in a total volume of 1.0 ml. Two milliliters of 3,5-dinitrosalicylate reagent were then added and the tubes were kept in a boiling waterbath for 30 min. The tubes were cooled and the volume of the contents made up to 10 ml with water and the color was read in a Klett colorimeter using filter No. 54. A standard curve was prepared with glucose. The procedure of Dahlquist (9) for measurement of glucose using Tris-glucone oxidase reagent could not be employed here, as there was inhibition of color formation. The inhibition was found to be due to saligenin formed in the reaction, reacting with the peroxidatic system.

Protein. Protein was determined by the procedure of Lowry et al. (11) using crystalline bovine serum albumin as standard.

Enzyme units. One unit of enzyme activity is defined as the amount of enzyme required to hydrolise 1 amole of substrate per minute at 37°.

Preparation of homogenate. Muscle homogenates from the small intestine of adult monkeys (Macaca mulatta) were prepared by homogenizing the intestinal mucosa with 4 vol of cooled 115% KCI in a Waring Blender for 3 min. The soluble and the particulate fractions were obtained from the crude homogenate by centrifuging at 105,000 × g for 1 hr at 5° in a preparative ultracentrifuge (Spinco model L). After separating the supernatant fluid, the particulate fraction was washed and suspended in 0.01 M K-phosphate buffer, pH 7.0, and made up to the original volume of the homogenate.

Purification of β-galactosidase from the supernatant fraction. All the operations were carried out at 0-5°. The supernatant fraction prepared as given above was used as the enzyme source.

Ammonium sulfate precipitation. The supernatant fraction (300 ml, 3.4 g protein) was brought to 50% saturation with ammonium sulfate by the slow addition of 87.3 g of the salt and the stirring
continued for 30 min after the last addition. The precipitate formed was discarded and the ammoo-
nium sulfate concentration in the supernatant fraction (30% ml) raised to 70% saturation by the
further addition of 38.8 g of salt and stirring for 30.

The precipitate obtained was collected by centri-
fugation and dissolved in about 30 ml of 0.01 m
phosphate buffer, pH 7.0, and dialyzed against
0.001 m phosphate buffer, pH 7.0, for 48 hr.

Treatment at pH 5.0. The pH of the dialyzed
ammonium sulfate fraction was brought to 5.0 by
careful addition of 1 m acetic acid. The precipita-
tion was removed by centrifuging at 10,000g for 20 min and
the pH of supernatant fraction was brought back to 7.0 by the addition of 1 m sodium
bicarbonate.

DEAE-Sephadex Chromatography. The super-
natant fraction (100 mg protein) from the previous
step was applied to a column of DEAE-Sephadex,
A-50 (resin bed, 1 X 12 cm) equilibrated with
0.01 m K-phosphate buffer. Elution was carried
out by a linear phosphate buffer, pH 7.0, gradient
(0.01-0.1 m) obtained by use of the Vari- 
sed de-
vise (Technicon). Fractions (2-ml) were collected.
The enzymatically active fractions (Fig. 1) were
pooled and concentrated by dialysis.

The enzyme was purified 44-fold with an overall re-
covery of 51% and was free of particulate lacticase
and particulate hetero-€-galactosidase activities.

A summary of the purification steps is given in
Table I.

TABLE I

| Purification of €-Galactosidase from the Supernatant and Particulate Fractions from Monkey Intestine |
|--------------------------------------------------|--------------------------------------------------|
| Supematent enzyme | Total units | Total protein (mg) | Activity (units/mg protein) | | Particulate enzyme | Total units | Total protein (mg) | Activity (units/mg protein) | | | | | |
| 100,000g supern-
| 104 | 3.447 | 0.63 | | 100,000g sediment | 17.4 | 2.169 | 0.008 | | | | | |
| 50-70% AmSO4 | 67 | 261 | 0.36 | | Triton ext. | 4.3 | 357 | 0.008 | | | | | |
| pH 5.0 treatment | 64 | 95 | 0.56 | | pH 5.0 treatment | 2.9 | 168 | 0.030 | | | | | |
| DEAE-Sephadex | 53 | 41 | 1.30 | | DEAE-Sephadex elution | 1.3 | 90 | 0.016 | | | | | |
| elution | | | | | Sephadex G-200 | | | 0.019 | | | | | | |

Fig. 1. DEAE-Sephadex chromatography of
monkey intestinal supernatant. €-Galactosidase,
€-Galactosidase (●-●); protein (□—□). €-
Galactosidase was assayed using o-nitrophenyl
€-galactoside as substrate.
The activity of hetero-β-galactosidase measured with o-nitrophenyl β-n-galactoside as substrate was found to be distributed both in the particulate (40%) and in the supernatant (60%) fractions obtained on centrifugation (100,000 g, 60 min) of the homogenate in KCl (1.15%).

The bulk of the lactase activity (92%) measured with lactose as substrate (pH 5.4) was also present in the particulate fraction and is in agreement with our earlier observation (4) on the behavior of lactase and hetero-β-galactosidase. When the washed particulate fraction was treated with buffers (0.01 M, K-phosphate, pH 7.0) the hetero-β-galactosidase or lactase was not released into the supernatant fraction. Treatment with Triton X-100 (0.1%) in phosphate buffer (0.01 M, pH 7.0) followed by sonication for 15 min (20 Kc) released a considerable proportion (25%) of the hetero-β-galactosidase activity and a small amount (12%) of lactase activity. Papain treatment used for the solubilization of lactase almost completely destroyed the above hetero-β-galactosidase activity.

Properties of the Purified Enzymes

pH Optima of particulate and supernatant hetero-β-galactosidase. Investigation of the pH optima of the purified supernatant and particulate hetero-β-galactosidase activities showed a marked difference. The particulate hetero-β-galactosidase showed optimal activity at pH 4.5 (Fig. 3) while the supernatant enzyme showed an optimum at pH 7.0 (Fig. 4). The results were verified by using buffers with overlapping pH values and mixed buffers and the pH values given correspond to the actual pH of the reaction mixture.

The solubilized particulate β-galactosidase exhibited a shoulder at pH 7.0 in the pH activity curve (Fig. 3). The pH 7.0 activity, presumably due to the supernatant enzyme, was removed during purification at the DEAE-Sephadex step and it gave a sharp optimum at pH 4.5. However, similar purification procedures did not abolish the shoulder at pH 4–5, in the pH activity curve obtained with the supernatant fraction or the purified enzyme (Fig. 4). In view of the clear separation between the supernatant and particulate β-galactosidases by DEAE-Sephadex fractionation, it is unlikely that the activity at pH 4–5 (Fig. 4) is due to contamination by the particulate enzyme. This
fact is further supported by the absence of lactose hydrolysis by the purified supernatant enzyme, while the purified particulate enzyme shows lactase activity.

A fresh surgical specimen of human intestine became available at this stage of the study and the pH optimum of the supernatant β-galactosidase was found to be 4.6 using either o-nitrophenyl, β-galactoside or 6-bromo-2-naphthyl β-galactoside as substrate (Fig. 5). There was no significant enzyme activity at neutral pH values and this property is in sharp contrast to that of the enzyme from monkey or rat intestine (17).

![Fig. 4. pH Optima of monkey intestinal "supernatant" β-galactosidase. Crude 100,000g supernatant (O—O); purified fraction (●—●). o-Nitrophenyl β-galactoside was used as substrate. For buffers used see legend in Fig. 3.](image)

![Fig. 5. pH Optima of human intestinal supernatant β-galactosidase. A 10% homogenate in 1.13% KCl was centrifuged at 100,000g for 60 min and the supernatant fraction was used for assay with o-nitrophenyl β-galactoside as substrate. 0.1 M Citrate-phosphate buffer (●—●); 0.05 M veronal-HCl buffer (O—O).](image)

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Particulate β-galactosidase</th>
<th>Supernatant β-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>o-Nitrophenyl β-galactoside</td>
<td>2.5</td>
<td>0.020</td>
</tr>
<tr>
<td>p-Nitrophenyl β-galactoside</td>
<td>0.6</td>
<td>0.050</td>
</tr>
<tr>
<td>p-Nitrophenyl β-glucoside</td>
<td>0.4</td>
<td>0.014</td>
</tr>
<tr>
<td>Phenyl β-galactoside</td>
<td>3.0</td>
<td>0.006</td>
</tr>
<tr>
<td>Phenyl β-glucoside</td>
<td>9.0</td>
<td>0.003</td>
</tr>
<tr>
<td>6-Bromo, 2-naphthyl β-galactoside</td>
<td>0.5</td>
<td>0.004</td>
</tr>
<tr>
<td>6-Bromo, 2-Naphthyl β-glucoside</td>
<td>0.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Lactose</td>
<td>50.0</td>
<td>0.020</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>—</td>
<td>22.0</td>
</tr>
</tbody>
</table>

* $V_{max}$ values are given as moles substrate split/min/mg protein. Assays were performed as given under Materials and Methods using optimal conditions.

**Substrate specificity.** A number of substituted β-galactosides and β-glucosides were used as substrates. The particulate and supernatant β-galactosidases hydrolyzed both β-galactosides and β-glucosides (Table II). p-Nitrophenyl β-galactoside was hydrolyzed faster than o-nitrophenyl β-galactoside or p-nitrophenyl β-glucoside, by the supernatant enzyme. Further, among the phenyl- and 6-bromo 2-naphthyl substituted substrates, β-glucosides were hydrolyzed faster than β-galactosides while the reverse was true with the particulate β-galactosidase. The $K_m$ and more especially the $V_{max}$ values for 6-bromo 2-naphthyl glucoside and galactoside differed significantly for the two enzymes. The supernatant β-galactosidase did not hydrolyze lactose while the particulate enzyme acted on this substrate at a rate equal to the hydrolysis of o-nitrophenyl, β-galactoside.

The rate of heat inactivation of particulate β-galactosidase obtained by DEAE-Sephadex and Sephadex G-200 chromatography was studied using both o-nitrophenyl...
Hydrolysis of lactose and lactose as substrates. The DEAE-Sephadex fraction contained traces of lactose (pH 5.4) and showed different rates of heat inactivation using lactose or o-nitrophenyl β-galactoside as substrates (Fig. 6). However, identical rates of heat inactivation were obtained for the Sephadex G-200 eluates with both substrates showing that the hydrolysis of lactose by the hetero-β-galactosidase fraction was not due to a contamination by the major intestinal lactase (Fig. 6).

Mixed substrate incubation. As both β-galactosidases act on β-galactosides and β-glucosides it was of interest to find out whether both substrates are split at the same site. The ratio of hydrolysis of p-nitrophenyl β-glucoside and β-galactoside was constant throughout purification in both cases. The ratio was 0.41 for the supernatant enzyme and 0.47 for the particulate enzyme.

To find out whether β-galactosides and β-glucosides act as competitive inhibitors for the hydrolysis of each other mixed substrate incubations as employed by Heyworth and Walker (12) were performed. Using the supernatant enzyme the hydrolysis of the corresponding substrates were studied separately and in a mixture. The amount of product formed (nitrophenol) when the substrates were together in a mixture was less than the theoretical amount (Table III). This suggested that both substrates compete for the same site on the enzyme for hydrolysis. Similar results were also obtained using the particulate enzyme (data not shown in table).

Effect of metal ions and p-hydroxymercuribenzoate. Several metal ions were tested for their effect on the enzyme activity (Table IV). Mn²⁺, Mg²⁺, and Ni²⁺ did not significantly affect the activity of either enzyme while Cu²⁺, Ag⁺, and p-hydroxymercuribenzoate were inhibitory. The extent of inhibition by Cu²⁺ or Ag⁺ was different for the two enzymes. The inhibition by p-
hydroxymercurobenzoate could be reversed by 8-mercaptoethanol. EDTA showed no inhibitory effect.

Effect of glucoson- and galactonolactones.
The use of sugar lactones in establishing bond specificity has been demonstrated by Conchief et al. (13), and Levvy et al. (14). The effect of glucoson- and galactonolactones was tried on the hydrolysis of p-nitrophenyl-8-galactoside and p-nitrophenyl 8-glucoside by both enzymes and the Kᵢ values obtained are given in Table V. Glucosonlactone was a powerful inhibitor compared to galactonolactone in the hydrolysis of the two substrates by both the enzymes. The inhibition of 8-glucoside hydrolysis was higher than that of 8-galactoside hydrolysis.

**DISCUSSION**
The present study provides suggestive evidence for the existence of two hetero-8-galactosidases in monkey small intestine.

**TABLE IV**

Effect of Metal Ions, P-Hydroxymercuribenzoate, and EDTA on Hetero-8-Galactosidases from Monkey Intestine[^1^]

<table>
<thead>
<tr>
<th>Addition</th>
<th>% Activity</th>
<th>Particulate enzyme</th>
<th>Supernatant enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Mg[^2^]</td>
<td>100</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>Mg[^3^]</td>
<td>100</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Ag[^4^]</td>
<td>39</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Ca[^5^]</td>
<td>54</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>10</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

[^1^] All substances were tested at a final concentration of 10^-4 M.

**TABLE V**

Inhibition of Monkey Intestinal Hetero-8-Galactosidases by Lactones

<table>
<thead>
<tr>
<th>Substrate used</th>
<th>Inhibitor</th>
<th>Kᵢ value(s) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Particulate 8-galactoside</td>
</tr>
<tr>
<td>p-Nitrophenyl 8-galactoside</td>
<td>Glucuron-8-lactone</td>
<td>6.0 × 10⁻³</td>
</tr>
<tr>
<td>p-Nitrophenyl 8-galactoside</td>
<td>Galactonom-γ-lactone</td>
<td>1.3 × 10⁻³</td>
</tr>
<tr>
<td>p-Nitrophenyl 8-glucoside</td>
<td>Glucuron-8-lactone</td>
<td>1.3 × 10⁻⁵</td>
</tr>
<tr>
<td>p-Nitrophenyl 8-glucoside</td>
<td>Galactonom-γ-lactone</td>
<td>0.5 × 10⁻²</td>
</tr>
</tbody>
</table>

The two hetero-8-galactosidases differ with respect to their cellular location, one of them being present in the particulate fraction while the other is in the supernatant fraction. The bulk of the lactase activity was also sedimented with the particulate fraction. The difference between lactase and hetero-8-galactosidase activities of the particulate fraction has already been shown (4). The particulate lactase has been purified from monkey intestine (15) after solubilization with papain and was found to contain only traces of hetero-8-galactosidase activity. The ratio of lactase to hetero-8-galactosidase activities in the purified lactase was found to be 15.

The hetero-8-galactosidases from the supernatant and particulate fractions differ from each other in their pH optima. The particulate enzyme showed a pH optimum of 4.5 while the supernatant enzyme was active over a wide range of pH with an optimum at pH 7.0. The two enzymes differ in their relative activities with different substrates and susceptibility to inhibition by heavy metal ions. The supernatant enzyme did not hydrolyze lactose while the particulate enzyme hydrolyzed lactose. This activity does not arise out of a contamination of the major intestinal lactase as shown by identical heat-inactivation rates of lactose and 8-nitrophenyl 8-galactoside hydrolysis.

The heterogeneity of intestinal 8-galactosidase has been studied in rat (1, 3, 16, 17), rabbit (1), and human (1, 18, 19) intestines. Two 8-galactosidase activities have been shown to be present, one in the particulate and the other in the soluble fraction. The particulate enzyme hydrolyzes lactose predominantly and is essentially a disacchari-
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dase (lactase). The soluble enzyme from the rat intestine predominantly hydrolyzes hetero-β-galactosides (pH optimum, 3.5) and can be subfractionated into two fractions which show identical kinetic behavior by DEAE-cellulose chromatography. Monkey intestine appears to be unique in that it contains two hetero-β-galactosidases one in the supernatant (pH 7.0) and the other in the particulate fraction (pH 4.5). In addition to the above two β-galactosidases, there is a lactase in the particulate fraction acting predominantly on lactose and which exhibits a pH optimum at 5.4. Lactase has been shown to be present in the brush border region of the intestinal epithelial cell (1, 20). The location of the particulate hetero-β-galacto-
idase has not been investigated and it is likely to be lysosomal in view of its low pH optimum and extraction with Triton X-100. The ratio of hydrolysis during purification, mixed substrate incubation, and the lactone-inhibition studies show that β-galactosides and β-glucoisides are hydrolyzed at the same site by both the enzymes in agreement with the earlier observations on the overlapping specificity β-glucoisidase and β-galactosidase in the intestine (21), liver (22), and kidney (23).

Absence of enhancement of activity by the added metal ions and lack of inhibition by EDTA suggest that there is no metal ion requirement for enzyme activity. This is in contrast to the property of bacterial β-
galactosidase (24, 25), which is activated by Mg²⁺. The inhibition of enzyme activity by p-hydroxymercuribenzoate and the reversal of the inhibition by 2-mercaptoethanol show that sulfhydryl groups are probably in-
volved in the catalytic activity.

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