Fluorescent Spot Test for Detecting Carbohydrate-Splitting Enzymes in Meconium

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I describe a fluorescent spot test for detecting the acidic glycosidases in meconium. Meconium is mixed with various 4-methylumbelliferyl glycosides at the appropriate pH. In the presence of carbohydrate-splitting enzymes, 4-methylumbellifere, a strongly fluorescent compound, is released. The reaction mixture, when spotted on chromatography paper and viewed under ultraviolet light, exhibits a bright fluorescence for specimens containing acidic glycosidases. The technique is applicable to any test that involves production of fluorescent products from non-fluorescent reactants. The procedure was applied to meconium specimens selected because of increased albumin content known to be associated with cystic fibrosis in newborns. The presence of β-D-fucosidase in meconium was correlated to increased albumin content. Detection of β-D-fucosidase by the present procedure may be useful as a screening test for cystic fibrosis.

Additional Keyphrases: cystic fibrosis · screening · heritable disorders · acidic glycosidases · enzyme stability · other applications of the technique

4-Methylumbelliferone (4MU), a strongly fluorescent compound, has been conjugated with a wide variety of carbohydrates to form 4-methylumbelliferyl glycosides, which have been used as substrates for determining acidic glycosidase activity (1). As catalyzed by carbohydrate-splitting enzymes, 4MU is released from 4-methylumbelliferyl glycoside, and the released product is detectable under ultraviolet light in alkaline solution.

Because of the commercial availability of these fluorescent glycosides to serve as glycosidase substrates, a simple and rapid test for detecting the presence of acidic lysosomal glycosidases in biological samples was developed in our laboratory. This communication describes the procedure and its application for testing various lysosomal glycosidases in meconium samples. The same procedure should be applicable to other types of tissue, such as serum, urine, leukocytes, or fibroblasts.

Materials and Methods

A "Chromato-Vue" viewing box (Ultraviolet Products Inc., San Gabriel, CA 91778) was used for detecting fluorescence. Sodium citrate, citric acid, and sodium carbonate, all ACS grade, were purchased from Fisher Scientific Co., Fair Lawn, NJ 07410. 4-Methylumbelliferyl-β-D-fucoside, α-D- and β-D-galactosides, and α-D- and β-D-glucosides were obtained from Sigma Chemical Co., St. Louis, MO 63178. Whatman No. 1 chromatography paper for spot test was purchased from Whatman, Inc., Clifton, NJ 07014. Albumin test strip was the product of Boehringer-Mannheim, Elk Grove Village, IL 60007. Meconium was collected from newborn units of local hospitals in a sealed plastic bag and frozen until tested. All 4-methylumbelliferyl glycoside substrate concentrations were 100 μmol/L, prepared in 200 mmol/L citrate buffer. The optimal pH for each glycosidase was adapted from: Barrett and Heath (1) for β-D-fucosidase (EC 3.2.1.38) and α-D-galactosidase (EC 3.2.1.22) the pH was 5.0, for β-D-galactosidase (EC 3.2.1.23) and α-D-glucosidase (EC 3.1.1.20) it was 4.0, and for β-D-glucosidase (EC 3.1.1.21) it was 5.5. The solution used to stop the reaction was 0.5 mol/L sodium carbonate, pH 10.8; it also served to intensify the fluorescence by virtue of its alkalinity.

Incubations were done in a Cooke disposable clear plastic plate with a U-shaped bottom well, the capacity of each well being 125 μL (Scientific Products, McGaw Park, IL 60085). The testing procedures for all glycosidases were basically identical except that a specific substrate was used for each enzyme. Each sample was determined in duplicate. Several large specimens were repeatedly frozen and thawed and retested more than 10 times during six months of storage, to determine the stability of the enzymes. The samples were thawed at room temperature immediately before the test. With a sharp-pointed straight forceps, about 2 mg of wet meconium (about pinhead size) was picked out of each bag, and placed into the bottom of a well. One drop (about 30 μL) of substrate-citrate buffer solution was added to each well. The incubation plate was covered with glass to prevent evaporation and placed in an incubator at 37 °C for various intervals, to determine an incubation period.

At the end of the incubation period, one drop (about 30 μL) of the pH 10.8 sodium carbonate solution was added to each well to stop the acidic glycosidase reaction and enhance the fluorescence of 4MU. About 5 μL of each incubation mixture was applied on Whatman No. 1 chromatography paper to make a 10-mm spot. After drying, the paper was placed in the Chromato-Vue box and examined under long-wave ultraviolet light in the dark room. The results were recorded by the tester according to the presence or absence of fluorescence. The spot with fluorescence was assigned as a positive (+), and the spot lacking fluorescence as a negative (−).

A quenching effect of meconium on fluorescence intensity was tested by selecting a pretested meconium sample devoid of β-D-fucosidase activity to serve as a basal masking medium. About 2 mg of negative meconium was placed into the bottom of each reaction well. Graded quantities of 4MU aqueous solution, ranging from 0 to 10 nmol in 30 μL, were added to each well; then, one drop of the pH 10.8 sodium carbonate solution was added. Procedures for paper chromatography and fluorescence examination were identical to those described in the previous section.

Results and Discussion

The chromatographic spots from meconium with glycosidase activities fluoresced brightly under the ultraviolet light, whereas the samples that were devoid of enzymes showed a stain mark with no fluorescence (Figure 1). The quenching effect of meconium on fluorescence is shown in Figure 2. The fluorescence of the 4MU-sodium carbonate mixture without meconium was visible at a concentration of about 0.1 nmol in 60 μL. When 2 mg of meconium was added to the same volume of 4MU-sodium carbonate mixture, the fluorescence became obvious only when the quantity of 4MU exceeded 0.4 nmol.
Accordingly, the sensitivity of the test for the enzymes was estimated to be equivalent to the activity of 0.2 unit, a unit being defined as 1 μmol of 4MU released per gram of fresh meconium under the present testing condition. This cutoff point, 0.2 unit, for distinguishing the positive from negative in the spot test was confirmed by quantitation of the enzyme activity by the procedure of Wood (2). Meconium specimens with 0.2 unit or greater β-D-fucosidase activity by the quantitative assay was readily detectable by the spot test, whereas activity of less than 0.1 unit was not detectable under ultraviolet light.

As illustrated in Figure 1, a 30-min incubation at 37 °C seemed sufficient to release 4MU from the substrates; longer incubation increased neither the intensity of fluorescence nor the proportion of positive samples. Samples judged to be negative after 30-min incubation were also negative after a 2-h incubation. Accordingly 30-min incubation was adopted as the standard testing condition.

Once the incubation mixture was spotted on chromatography paper, the fluorescence remained indefinitely. This stability of the fluorescence on paper allows a permanent record of test results.

In the present study, no sophisticated machinery was involved. The reagents for the test are simple. The citrate buffers, stored in a refrigerator, and the substrate solutions, stored in a freezer, were stable for at least six months. 4-Methylumbelliferon glycoside substrates were not fluorescent nor were they susceptible to autodegradation during the incubation. Therefore, no product separation is required after the enzyme reaction. The testing procedure is rapid, highly reproducible, and relatively inexpensive.

All of the acidic glycosidases tested appeared to be very stable: after freezing and thawing 10 times or more, the enzymes were still active, and samples dried at room temperature for several days exhibited the same activity as when freshly collected. Two milligrams of meconium from each specimen was smeared on filter paper and air dried for two days. The smeared area was cut with a hole puncher to obtain a disc 6 mm in diameter. When the dried meconium on paper was tested against the fresh sample, the fluorescence intensity was the same for both.

Table 1 shows the frequency of positive spot tests for various acidic glycosidases in the meconium. All the meconium specimens used for the lysosomal glycosidase tests were first screened for albumin by use of Boehringer-Mannheim albumin test strips (3). More than 70% of the albumin-positive specimens showed a positive β-D-fucosidase reaction; in contrast, only 6% of the albumin-negative specimens had detectable β-D-fucosidase activity. However, 95–97% of the meconium specimens exhibited significant α-D-glucosidase activity, regardless of albumin content in the meconium. I found no α- and β-D-galactosidases and β-D-glucosidase in any meconium sample that gave a negative reaction to the albumin test. On the other hand, some 6 to 12% of the albumin-positive specimens, showed a remarkable activity of these enzymes.

Detection of an increased albumin content in meconium has been used as a means of screening for cystic fibrosis in newborns (3). Shwachman et al. (4) and Antonowicz et al. (5) recently found a high β-D-fucosidase activity in meconium specimens from cystic fibrosis subjects. The present study shows that a positive β-D-fucosidase test is correlated with an increased albumin content in meconium specimens. Testing for the presence of β-D-fucosidase in meconium seems to be an appropriate approach to screening cystic fibrosis, as has been suggested (4, 5). However, in the study by Shwachman et al. (4), p-nitrophenyl glucoside was used as a substrate. The p-nitrophenol liberated by the enzymatically catalyzed reaction showed a yellow color in alkaline solution. This yellow color would be difficult to distinguish from the color of meconium in the incubation mixture. The present fluorescent procedure would have the advantage that the fluorescence of positive samples is easily distinguishable; the yellow color of meconium does not interfere. Taking advantage of the great stability of meconium β-D-fucosidase, the present method

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**Table 1. Frequency of Positive Fluorescent Spot Test for Acidic Glycosidases in Relation to Presence of Albumin in Meconium**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Frequency of positive glycosidases (no. pos./no. tests)</th>
<th>Albumin present, %</th>
<th>Albumin absent, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-Fucosidase (EC 3.2.1.38)</td>
<td>244/337</td>
<td>72</td>
<td>95/1490</td>
</tr>
<tr>
<td>α-D-Galactosidase (EC 3.2.1.22)</td>
<td>10/96</td>
<td>10</td>
<td>0/96</td>
</tr>
<tr>
<td>β-D-Galactosidase (EC 3.2.1.23)</td>
<td>6/96</td>
<td>6</td>
<td>0/96</td>
</tr>
<tr>
<td>α-D-Glucosidase (EC 3.1.1.20)</td>
<td>93/96</td>
<td>97</td>
<td>9/98</td>
</tr>
<tr>
<td>β-D-Glucosidase (EC 3.1.1.21)</td>
<td>12/96</td>
<td>12</td>
<td>0/96</td>
</tr>
</tbody>
</table>

* Meconium albumin was tested for by use of the Boehringer-Mannheim strip
would be suitable for establishing a cystic fibrosis screening program in central laboratories.

The technique described above should also be applicable to other processes that involve release of fluorescent products from non-fluorescent reactants. A few examples are: the tests for activities of trypsin (6) and arginine esterase (7) by 4-methylumbelliferyl-p-guanidinobenzoate, of lysosomal hydrolases by 4-methylumbelliferoylglycosides (8), of albumin by 8-anilino-1-naphthalene sulfonic acid (9), and detection of a wide variety of drugs, such as amphetamines by dihydrodutidine derivative (10), salicylamide by alkalization (11), and warfarin by acetone-acidification (12) in biological fluids.

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References