Development of an Agarose Gel Electrophoresis Technique for Determining α-Amylase Isoenzymes

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Evaluation of α-amylase isoenzymes as a clinical diagnostic aid by previous methodologies has been either too insensitive or too cumbersome for routine clinical laboratory use. With use of an agarose gel electrophoresis system (Worthington Diagnostic Systems, Inc.) serum amylase activity can easily be resolved into at least nine isoenzymes, the resolution being comparable with that of isoelectric focusing. Samples can be analyzed in less than one working day, with use of conventional reagents.

Additional Keyphrases: saliva · pancreatic extracts · isoelectric focusing compared · reference interval · hyperamylasemia · pancreatitis

Hyperamylasemia has been associated with some clinical disorders of both pancreatic and nonpancreatic origin (1). Their differentiation, based upon determination of serum lipase or amylase/creatinine clearance ratio, or both, has yielded inconsistent results (1–3). A method of separately quantifying pancreatic (P) and salivary (S) isoenzymes of amylase might therefore be clinically desirable.

Two major and two to five minor amylase isoenzymes have been described in human serum (4–6). Salivary amylase accounts for approximately 60% of the total serum activity and consists of at least four separate isoenzymes (4, 6). Pancreatic amylase accounts for the remaining 40% and contains at least three isoenzymes (4, 6–8). Of the numerous methods used to determine amylase isoenzymes, only one is commercially available, and it detects only total P and total S activities (9). Isoamylase determination by cellulose acetate electrophoresis resolves amylase activity into two major and two minor isoenzymes (8, 10). In this system, however, a potentially important minor P isoenzyme (P2) migrates close to the major S band, which may lead to difficulties in interpretation (11, 12). Column isoelectric focusing (13) and polyacrylamide gel electrophoresis (PAGE) (14) are extremely sensitive methods but are time consuming and impractical for routine use. An ideal assay, therefore, should resolve salivary and pancreatic amylase into both major and minor isoenzymes; be sensitive and reproducible; and be easily adapted to a clinical biochemistry laboratory. On the basis of the work of Leclerc and Forest (4) we have devised a method of agarose gel electrophoresis that we believe meets this criteria.

Materials and Methods

Preparation of salivary and pancreatic extracts. Saliva from healthy volunteers was clarified by centrifugation (2000 × g, 25 min), and stored in portions at −20 °C. Normal pancreatic tissue obtained at autopsy was homogenized in cold 9.0 g/L sodium chloride solution, centrifuged (2500 × g, 20 min), and the supernate stored in portions at −20 °C. The aliquots were diluted with saline before electrophoresis.

Preparation of serum samples for total amylase. Total serum activity was assayed within 4 h of blood sampling, by use of the "Phadebas Amylase Test" (Pharmacia Diagnostics, Piscataway, NJ 08854). After analysis the samples were stored at −20 °C until isoenzyme assay.

Preparation of serum samples for isoelectric focusing. Serum, saliva, and pancreatic extracts, 200 μL of each, were dialyzed overnight against distilled water. The dialyzed material was then concentrated to about 50 μL with polyacrylamide gel (Lyphogel; Gelman Science, Inc., Ann Arbor, MI 48106). We used 20 to 40 μL of the concentrate for isoelectric focusing.

Preparation of the wheat-protein inhibitor of salivary isoamylase. The wheat-protein inhibitor of salivary isoamylase was prepared according to the method of O’Donnell and McGenney (15), omitting the Sephadex G-50 gel-filtration step. We determined inhibitor activity by using the prepared salivary and pancreatic extracts. A working inhibitor solution for use with isoelectric focusing and agarose gel electrophoresis was freshly prepared by diluting the stock inhibitor with a solution containing 9.0 g of NaCl and 5.0 g of albumin per liter.

Isoelectric focusing on polyacrylamide gels, 50 g/L. We electrofocused 20–40 μL of sample dialysand on LKB Ampholine PAGplates (pH 5.5–8.5; cat. no. 1804-103) with the LKB Multiphor Model 2103 apparatus (LKB, Gaithersburg, MD 20877). The gel concentration (T) was 50 g/L, the degree of crosslinkage (C) 3%, and the ampholine concentration 22 g/L. Samples were electrofocused for 2.5 h at settings of 10 °C, 25 W, 1600 V, and 50 mA. pH gradients were checked at room temperature after each run.

Electrophoresis on agarose gels, 10 g/L. Serum and pancreatic and salivary extracts were electrophoresed with the "Panagel" system (cat. no. DR0047600; Worthington Diagnostics Systems, Inc., Freehold, NJ 07728). We soaked these agarose gels overnight in Tris–borbital buffer (pH 8.8, 60 mmol/L, High Resolution Buffer; Gelman Science), which we also used as the electrophoresis buffer—and which could be used for four runs if the electrode poles were reversed after each run. Sample volume was 20 μL for samples with total amylase values <1000 U/L; samples with greater activity were appropriately diluted. The electrophoresis was at room temperature (20 °C) for 2 to 2.5 h.

Staining of amylase isoenzymes on agarose and polyacrylamide gels. Amylase isoenzymes were made visible by overlaying the gels with a suspension of eight Phadebas Amylase Test tablets dissolved in 10 mL of the albumin–saline solution and leaving the overlaid gels for 2.5 to 3 h at 56 °C, in a moist chamber. After staining, each gel was rinsed with distilled water to remove residual stain. The agarose gel was then soaked in ethanol for at least an hour (usually overnight), blotted, and dried in an oven at 56 °C. The polyacrylamide gel was soaked in a preserving mixture containing, per liter, 100 mL of glycerol, 250 mL of metha-
Results

Identification and Nomenclature of Amylase Isoenzymes

We identified the isoamylase bands on agarose gels by electrophoresing human pancreatic extract, saliva, and selected hyperamylasemic patients' sera in parallel (Figure 1). These specimens exhibited two major and as many as seven minor isoamylase bands. The major bands were easily identified by the migration of pancreatic extract and salivary isomylases. We identified the minor bands by two techniques. First, a wheat-protein inhibitor (15), which preferentially inhibits salivary amylase activity, was applied to the gel; the activities measured were compared with the isoamylase activities of the same samples overlaid with the albumin–saline solution. Using this technique, we noted a marked decrease in the relative activity of one major and three minor bands, which we designated here as salivary-derived bands. We also assayed pancreatic extract, parotid gland extract, and clarified saliva by isoelectric focusing (Figure 2). The pancreatic extract gave six isoenzyme bands with pI values of 7.5, 7.4, 7.0 (major), 6.1, 5.9, and 5.6. Parotid gland extract and clarified saliva yielded five isoenzyme bands with pI values of 6.7, 6.3 (major), 6.0, 5.7, and 5.6. We also used isoelectric focusing to assay hyperamylasemic patients' sera, which exhibited various combinations of major and minor bands on agarose gel electrophoresis (Figure 1), and matched the relative activities of each isoamylase by the two methods.

Following the convention of Leclerc and Forest (4), we designated the agarose gel migration pattern from cathode to anode as: P1, P1b, P2 (major), S1, P3, S2 (major), P4, S3, and S4. Because the relative electrophoretic mobilities may vary with different systems used, it is essential to confirm by pl measurement the identification of each isoenzyme, so there will be consistent reporting and interlaboratory comparison of the isoamylase patterns. We therefore established the isoelectric point of each isoenzyme resolved in this agarose gel system. Pancreatic extract was electrofocused and the individual bands were eluted with albumin–saline solution, concentrated with Lyphogel, and then electrophoresed on the agarose gel. Parotid extract was electrophoresed on the agarose system and the bands were eluted in albumin–saline solution, concentrated with Lyphogel to 50–100 μL, and then electrofocused. These bands were stained for activity and, with the silver stain, for protein. We were thus able to identify the pl of each isoenzyme. These are as follows: P1 7.5, P1b 7.4, P2 7.0, P3 6.1, P4 5.9, S1 6.7, S2 6.3, S3 6.0, and S4 5.7.

Method Evaluation

Sensitivity. Pancreatic and salivary amylase could be demonstrated in samples having total activity >30 U/L, the lowest activity measurable. Any isoenzyme activity exceeding 5 U/L could be quantified.

Precision. To evaluate within-run variation, we analyzed 14 aliquots of each of three sera containing various pancreatic and salivary isoenzyme activities (Table 1). Between-run variation was assessed by analyzing three other samples with P2, P3, S2, and S3 activity in duplicate on 14 consecutive gels. Table 2 illustrates the between-run precision for one of these samples.

Linearity. Salivary and pancreatic amylase extracts were serially diluted and their total amylase activity was assayed in triplicate. Mixtures of pancreatic and salivary extracts were then prepared and analyzed for total amylase activity and isoenzyme composition by electrophoresis. The proportions of S and P (in percent) were calculated and compared with expected values. Salivary and pancreatic activity was not linearly related to absorbance. A logarithmic first-order equation, ln(activity) = A + B + C·lnA, best described the curve. Linear regression analysis of expected vs observed activity was applied to each component isoenzyme (r = 0.969). At low P/S ratios, P-type activity is overestimated by 5 to 7%. The overestimation of P-type activity at low P/S ratios has been attributed to the greater activity of pancreatic amylase than salivary amylase on the insoluble starch substrate (4, 5).

Isoenzyme patterns for normal sera. We evaluated the isoamylase pattern for samples from apparently healthy volunteers: seven men and seven women, ages 23 to 52 (mean 33.7) years (Table 3). The most common patterns observed were P2, S2, S3 in eight; P2, S2 in three; P2, P3, S2, S3 in two; and P1b, P2, S2, S3 in one. No age- or sex-related differences in the isoenzyme pattern were apparent.

Discussion

The nine isoamylase bands that can be identified may represent distinct isoenzymes of α-amylase, in vitro arti-
Table 1. Within-Run Variation of Isoenzyme Determinations in Three Sera

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(185 U/L, n = 14)</td>
<td>(785 U/L, n = 15)</td>
<td>(848 U/L, n = 14)</td>
</tr>
<tr>
<td>P2</td>
<td>S2</td>
<td>P2</td>
</tr>
<tr>
<td>X, %</td>
<td>37.3</td>
<td>63.4</td>
</tr>
<tr>
<td>SD, %</td>
<td>2.6</td>
<td>3.6</td>
</tr>
<tr>
<td>CV, %</td>
<td>6.9</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Total amylase activity, and no. of determinations.

Table 2. Between-Run Variation for a Serum Containing Total Amylase Activity of 564 U/L (n = 14)

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>P1b</th>
<th>P2</th>
<th>P3</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>X, %</td>
<td>40.0</td>
<td>8.1</td>
<td>40.2</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>SD, %</td>
<td>4.1</td>
<td>2.7</td>
<td>3.8</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>CV, %</td>
<td>10.2</td>
<td>9.4</td>
<td>9.4</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Reference Intervals for Amylase Isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>P1b</th>
<th>P2</th>
<th>P3</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.9</td>
<td>40.9</td>
<td>0.56</td>
<td>53.8</td>
<td>3.5</td>
</tr>
<tr>
<td>SD</td>
<td>3.5</td>
<td>9.2</td>
<td>1.4</td>
<td>9.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Range</td>
<td>0-13</td>
<td>26-59</td>
<td>0-4.1</td>
<td>39-74</td>
<td>0-8.8</td>
</tr>
</tbody>
</table>

Activity, U/L

| Mean | 1.5 | 66 | 1.2 | 84.8 | 5.5 |
| SD | 5.5 | 27.2 | 3.1 | 25.0 | 5.3 |
| Range | 0-20.5 | 25-114 | 0-7.6 | 24-123 | 0-17.6 |

*Range for total amylase activity, 56-232 U/L (mean 160, SD 47.3, n = 14).

facts, or genetic (phenotypic) variants. That these represent artifacts is improbable: first, the samples were aliquoted and frozen within hours of blood sampling, and, second, there was no significant day-to-day variation in the banding pattern when the same sample was consecutively assayed, which suggests that in vitro interconversion of isoenzymes at -20°C is probably negligible. However, recent evidence (16) suggests that amylase is a product of two genes (Amy1 and Amy2) with multiple alleles at each locus. Cathodally migrating isoenzymes such as P1 and P1b may represent phenotypic variants of pancreatic isoamylase, but most of the minor amylase isoenzymes are probably products of post-translational deglycosylation or deamidation, or both (16). Such changes would result in faster (anodal) migrating isoenzymes and would be consistent with the pattern of migration of the minor amylase isoenzymes in the present study. Whether the minor isoenzymes represent post-translational alterations or genetically distinct enzymes, this does not detract from their potential clinical significance.

With the thin-layer isoelectric focusing system (LKB Amphotline PAG, pH 5.5–8.5) that we used as a standard to identify the isoamylase bands obtained by agarose gel electrophoresis, we could resolve at least 11 distinct isoenzymes. The pi values for these isoamylases compare well with those reported hitherto (13, 14).

The nine isoenzymes seen on the agarose system have been identified by wheat-protein inhibition of salivary-type amylase and by pi determination. These two methods permit us to establish criteria by which the isoenzymes can be accurately identified. This becomes increasingly important as new methods for separating amylase isoenzymes appear and as the clinical importance of the minor bands (7, 11, 17, 18) emerges.

Following the suggestion of others (4), we labeled the major salivary band S2 and the major pancreatic band P2. This necessitated our designating the two most cathodolic pancreatic bands as P1 and P1b.

Our reference limits for the percentages of salivary and pancreatic fractions compare well with those reported (4, 19–21). The previously unreported isoenzyme we demonstrated, P1b, appeared in six of the 96 hyperamylasemic (total amylase >300 U/L) patients we evaluated. The prevalence of this band in normoamylasemic persons is unknown.

The one macroamylasemic specimen demonstrated the characteristic "blurring pattern" described by Leclerc and Forest (4), who also used agarose gel.

In comparison with other techniques, electrophoresis with agarose gel offers several distinct advantages for separating amylase isoenzymes. Although cellulose acetate electrophoresis has been advocated for the routine assay of serum isoamylases (22), being simple to perform and requiring only readily available equipment, resolution is generally poor and a potentially important minor P isoenzyme (P3) migrates very close to the major S band. This may lead to difficulties in interpretation, especially when the proportion of P3 is abnormally high. The more sophisticated techniques for amylase isoenzyme separation—polyacrylamide gel electrophoresis (13) and column isoelectric focusing (14)—have generally been relegated to the research laboratory. Both are time consuming, expensive, and relatively limited in the number of samples that can be assayed per run. These methods do, however, yield excellent separation of isoamylases and serve as standards with which to compare new methodologies.

Pharmacia Diagnostics, Inc., has recently released a commercial kit, the "Phadebas Isoamylase Test," for separation of serum amylase into total and pancreatic (P) isoamylase activities. It is based upon the principle that wheat-protein preferentially inhibits salivary (S) isoamylase, inhibition being 100-fold that for P isoamylase (15, 23). Although previous studies have demonstrated that this technique is simple and accurate over a defined range of serum amylase activities, there may be difficulties in interpreting very low, very high, and macroamylasemic specimens (5, 19, 24). In addition, that method gives no information concerning the relative activities of the minor amylase isoenzymes, which may be clinically important (17, 18).

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References


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