Pancreatic Amylase Measured in Serum by Use of a Monoclonal Antibody Immunochemically Immobilized to a Solid Phase

Theodore E. Mifflin, Marilyn Hamilton, Elizabeth Hubbard, Michael J. Kline, and David E. Bruns

We studied a method for measuring the pancreatic isoenzyme of amylase (EC 3.2.1.1) by use of a mouse monoclonal antibody against human salivary-type amylase (Clin Chem 1985;31:1283) coupled indirectly to particles of polyvinylidene fluoride via polyclonal goat anti-mouse immunoglobulin. These particles, in 200 μL of a suspension, could remove salivary amylase (activity 2200 U/L) from an equal volume of serum in 5 min. Measurement of amylase activity in the supernatant fluids from treated sera thus provided an assay of pancreatic amylase. Precision studies at three activity concentrations yielded within-run CVs of 1.6% to 1.7% (n = 25) and total CVs of 2.2% to 5.1% (20 days). Salivary amylase added to each of 10 sera was completely (99.8%, SD 1.6%) removed. The new method (y) showed the following regression statistics when compared with an electrophoretic method (x): slope = 0.989 (SD 0.019), intercept = -0.220% (SD 1.48%), SEE 4.0%, n = 51. Similar respective regression values were found for urine samples: slope = 0.934 (SD 0.053), intercept = -2.3 U/L (SD 3.2), SEE 8.4 U/L, n = 216. The following respective values were found when the new method (y) was compared with the previously described immunoprecipitation assay (x): slope = 1.02 (SD 0.02), intercept = 2.2% (SD 1.4%), SEE 3.3%, n = 23 sera. Reference intervals for pancreatic amylase activity in serum were established for three different substrates: maltotraose, maltopentaose, and p-nitrophenylheptaoside.

Additional Keyphrases: isoenzymes · monoclonal antibodies · immunoprecipitation assay · defined substrates · electrophoretic mobilities · urine

The diagnostic utility of amylase, long recognized (1), has motivated development of a wide variety of methods for measuring amylase activity. The goal of specifically measuring the pancreatic isoenzyme of amylase has stimulated the development of isoenzyme-specific assays (2) as well. Methods used in the specific assays include electrophoresis in its numerous forms (3), selective inhibition by plant proteins (4), and antigenic recognition. In the last category, several immunosassays involving polyclonal antisera have been reported (5–11), but most antisera have shown cross-reactivity between pancreatic and salivary amylases—not surprising, because the two isoenzymes are remarkably similar in amino acid sequence (>94%), molecular mass, and isoelectric point (2, 12).

The specific recognition of the salivary amylase isoenzyme by monoclonal antibodies has been reported (13–17). Several methods (18–21) exploit this specificity. Recently, we described an anti-salivary amylase antibody and the feasibility of using this antibody to measure amylase isoenzymes (18). Here we describe the clinical and laboratory performance of an assay in which this antibody is used. In this assay, the antibody has been coupled to a polymer through an immunochromenic attachment.

Materials and Methods

Amylase activity. We quantified amylase activity with three defined-substrate methods. Except as noted, we used an assay (22) supplied by Roche Diagnostics Systems, Nuttery, NJ 07110, with maltotraose as the substrate. In the two other assays, p-nitrophenylheptaoside (23) and maltopentaose (24) are used as substrates; these assay kits were from Boehringer Mannheim Diagnostics, Indianapolis, IN 46250, and DuPont, Wilmington, DE 19898, respectively. With the latter substrate, amylase activity was quantified in a two-point kinetic assay in an acu III (DuPont).

The maltotraose assay involved multi-point kinetics. It was done in the Cobas-Bio centrifugal analyzer (Roche) at 37 °C; the sample volume being 10 μL, diluent volume 30 μL, reagent volume 170 μL, delay time 270 s, and a wavelength setting 405 nm.

Glucose measurement. Serum glucose was quantified by use of a hexokinase-coupled assay (25) as described earlier (18) in a serum-blanked mode.

Preparation of purified salivary and pancreatic amylases. Both amylase isoenzymes were isolated in their purified forms as described earlier (26) and stored at −70 °C until needed.

Electrophoretic assay of amylase isoenzymes. Amylase isoenzymes were separated on Mylar-backed 10 g/L agarose films as described before (18, 27), with minor modifications. We increased from 37 °C to 45 °C the temperature at which the agarose films were incubated with the Phadebas suspension, and we used a newer-model integrating densitometer (Appraise: Beckman Instruments, Fullerton, CA 92634). We quantified pancreatic and salivary fractions by delimiting each amylase electrophotogram as guided by comparison with samples of purified amylase run concurrently as markers.

Directly immobilized anti-salivary amylase monoclonal antibody (DIAA). We directly immobilized antibody to salivary amylase (14) onto polyvinylidene fluoride particles (18). The suspension was sonicated (Branson Sonicator, Shelton, CT) at room temperature for 10 min, centrifuged at 1000 × g, then resuspended in storage buffer (see below).

Indirectly immobilized anti-salivary amylase antibody (IIAA). Goat anti-mouse immunoglobulin was first bound to particles of polyvinylidene fluoride according to a previous procedure (28), and antibody to salivary amylase (14) was then immobilized indirectly. The IIAA suspension was obtained from Roche. Suspensions of IIAA and DIAA were stored at 4 °C in a Tris buffer (20 mmol/L, pH 7.5) that contained, per liter, 1 g of sodium azide and 5 g of bovine serum albumin.

Pancreatic amylase immunoassay. Gently resuspend the particle suspension at room temperature by magnetic stir-

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rings for 15–20 min before use. Pipet 200 μL of serum into a 12 × 75 mm tube labeled "Pancreatic." Add an equal volume of IIAA suspension to the tube, vortex-mix briefly, and leave at room temperature for 5 min. Centrifuge the tube (1000 × g) for 10 min and determine the pancreatic amylase activity in the supernate. Measure the total amylase activity in a separate aliquot of the sample. Correct the amylase activity in the "Pancreatic" tube for dilution by multiplying by 1.9. (Use 1.9, rather than 2.0, because the particles occupy a portion of the 200 μL of IIAA suspension added to the serum sample; this nonpartitionable volume does not dilute the amylase activity and therefore leads to a slight enrichment of activity.)

The value of 1.9, provided by the manufacturer, was verified by the following procedure: Treat 10 randomly selected sera with IIAA particle suspension as described above. Quantify glucose in the supernatant fluids and in aliquots of corresponding untreated sera. Calculate for each sample the ratio of serum glucose to glucose in the supernate, then average the results for all 10 samples. We found a factor of 1.86 (SD 0.01) for the lot of IIAA suspension that we used, similar to results we have determined for previous batches of DIAA suspension prepared in-house (data not shown) and to the value 1.89 that can be calculated from our earlier publication on DIAA (18).

Controls. We prepared three separate pools of serum controls (Level I, Level II, and Level III) by mixing different activities of purified amylases with Fisher Control Serum I (Fisher Diagnostics, Malvern, PA 21046). Aliquots (0.5 mL) of each pool were stored at −70 °C, then thawed once, used, and discarded. Day-to-day precision data were collected on 20 days during a 26-day period. Electrophoresis controls contained either purified salivary amylase or a mixture of purified pancreatic and salivary amylases at 700–900 U/L activity and were included in all electrophoretic plates.

Capacity of IIAA suspension for salivary amylase. To experimentally establish the capacity of the IIAA suspension for salivary amylase, we added purified salivary amylase, 1134 U/L, to normal serum, then titrated aliquots of this mixture with decreasing volumes of IIAA suspension. Following incubation, we centrifuged the samples, analyzed the supernates by electrophoresis, and quantified their residual amylase activity.

Patients' samples. Sera with increased concentrations of total amylase were obtained from the Clinical Chemistry Section, University of Virginia Hospital Clinical Laboratories, as leftover samples after routine analyses were completed. These samples were analyzed by DIAA and IIAA methods and by electrophoresis. Urine samples were obtained from volunteers or were also leftover specimens from the clinical laboratory.

We defined three diagnostic groups, based on their clinical histories, which were obtained retrospectively by chart review. Acute pancreatitis was diagnosed in 10 patients (Group 1). Associated conditions included ethanol abuse in two patients, and one patient each with gallstones, pancreatic cancer, pancreatic pseudocyst, chronic pancreatitis, ileus, or papillotomy with endoscopic retrograde cholangiopancreatogram. Postoperative hyperamylasemia (Group 2) was present in five patients, three of whom had undergone cholecystectomy, one a partial colectomy (diverticulitis), and the other an anastomosis after a motor vehicle accident. The remaining five patients (Group 3) had diagnoses of cholecystitis, gastritis/duodenitis with or without peptic ulcer, pneumonia with ethanol withdrawal, and metastatic disease from an unknown primary cancer (this patient had a history of ethanol abuse).

Reference-interval estimation. Sera were collected from apparently healthy fasting volunteers, then stored at −20 °C. We fractionated the amylase isoenzymes by the Roche immunochemical method and quantified the amylase activities by the three defined-substrate methods. Boundaries of the reference intervals were estimated nonparametrically.

Results

Binding capacity of the immobilized antibody suspension. Figure 1 illustrates the capacity of the IIAA particle suspension to capture purified salivary amylase activity. Lanes 2–4 indicate that all of the added salivary amylase has been absorbed; a trace amount of salivary amylase can be detected in lane 5. The estimated capacity of a 200-μL suspension for salivary amylase (95% capture) therefore appears to exceed 2200 U/L.

Precision. Table 1 reports the precision of the indirect-coupled assay. With two exceptions, the CVs are all <2.6%. Both day-to-day and within-run precision of the assay remained essentially constant, regardless of the relative ratio of pancreatic amylase to salivary amylase. These precision results agree well with the values we previously observed (18) and also with precision data reported elsewhere for another amylase immuno precipitation assay (21).

Accuracy. Table 2 lists the fraction (%) of added salivary amylase removed from 10 sera. Added salivary amylase, 544 U/L, was completely (99.8%, SD 1.6%) removed. Comparing the results of the new assay with electrophoretic results for 51 sera showed excellent agreement (Figure 2). Plotting results from patients with different diagnoses showed no noticeable bias (see legend to Figure 2).

Comparison of the results from this regression with those published earlier (18) suggest similar responses of the IAA and DIAA methods. For a separate group of 23 samples we compared the results from the IIAA method with those from the DIAA method. When the IAA % pancreatic amylase results (y) were compared with the corresponding DIAA % pancreatic amylase results (x) obtained from 23 sera, excellent agreement was again observed: slope = 1.019 (SD 0.023), intercept = 2.16% (SD 1.4%), r = 0.9947, SEE = 3.3% (x ranged from 8% to 88%).

Electrophoretic analyses of both untreated and the corresponding IAA-treated supernates are illustrated in Figure 3. Note that a portion of the apparent pancreatic amylase activity (shaded areas) frequently comigrated with the salivary amylase marker (indicated by the horizontal bar).

Fig. 1. Determination of salivary amylase binding capacity

From left to right: Lane 1, purified salivary amylase marker. Lanes 2–8, decreasing volumes of IIAA suspension (200, 100, 50, 10, 5, 2.5, and 0 μL, respectively) were mixed with 200 μL of normal serum that contained purified salivary amylase, 1134 U/L, treated as described in the text, and the supernates were subjected to electrophoresis in agarose. Amylase activity was made visible as described. P, pancreatic; S, salivary-type amylases

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Table 1. Precision Studies

<table>
<thead>
<tr>
<th></th>
<th>Total amylase, U/L</th>
<th>Pancreatic amylase, U/L</th>
<th>Pancreatic fraction, %</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
</tr>
<tr>
<td>Within-run ( (n = 25) )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level I</td>
<td>141</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Level II</td>
<td>345</td>
<td>3.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Level III</td>
<td>651</td>
<td>7.4</td>
<td>1.1</td>
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<tr>
<td>Day-to-day ( (n = 20) )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level I</td>
<td>144</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Level II</td>
<td>341</td>
<td>6.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Level III</td>
<td>651</td>
<td>9.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Similar results were observed for other patients' samples assayed in this study.

Figure 4 illustrates that the IIAA procedure removed salivary amylase from urine as well. Comparison of the immunochemical \( y \) and electrophoretic \( x \) results (pancreatic amylase, U/L) from 26 urines yielded the following regression statistics: slope = 0.953 (SD 0.063), intercept = 2.3 (SD 3.2) U/L, SEE = 8.4 U/L, \( r = 0.963 \).

Reference interval studies. Table 3 lists the mean values, standard deviations, and reference intervals for total amylase, pancreatic amylase, and \% pancreatic amylase, as determined with the IIAA reagent. Reference intervals obtained with the IIAA suspension and maltopentaose and \( p \)-nitrophenylheptaoside substrates are also listed in Table 3. For 64 urine samples, the mean (and SD) pancreatic amylase was 57\%(16\%).

Clinical studies. Serum pancreatic amylase was increased in each of the 10 patients with acute pancreatitis (Group 1). The mean peak pancreatic amylase was 928 U/L (range 153–3570 U/L). In this group, the percent pancreatic amylase was also increased (mean 90\%, SE 2.5\%, range 80\%–100\%). Lesser increases of serum pancreatic amylase were seen in the Group 2 patients (post-operative hyperamylasemia): the mean peak values were 157 U/L (SE 25 U/L, range 90–244 U/L) and 82\% (SE 4\%, range 60–96\%). Similar values (104–552 U/L, 50–89\%) were found in four of the five patients in Group 3. In the remaining Group 3 patient, who had pneumonia and ethanol withdrawal, the pancreatic amylase activity was 33–34 U/L, 14–15\% of total amylase (n = 2). All values were confirmed by electrophoresis as shown in Figure 2.

Table 2. Removal of Salivary Amylase from 10 Sera

<table>
<thead>
<tr>
<th>Amylase activity, U/L</th>
<th>Before salivary amylase added</th>
<th>After salivary amylase added</th>
<th>Salivary amylase removed, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Pancreatic</td>
<td>Total</td>
</tr>
<tr>
<td>32</td>
<td>20</td>
<td>20</td>
<td>572</td>
</tr>
<tr>
<td>52</td>
<td>26</td>
<td>26</td>
<td>592</td>
</tr>
<tr>
<td>60</td>
<td>37</td>
<td>37</td>
<td>600</td>
</tr>
<tr>
<td>63</td>
<td>28</td>
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<td>80</td>
<td>52</td>
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</tr>
<tr>
<td>Mean</td>
<td>54</td>
<td>30</td>
<td>595</td>
</tr>
<tr>
<td>SD</td>
<td>14</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>

*Salivary amylase added = 544 U/L, final concentration.

Fig. 2. Comparison of pancreatic amylase results (\% \%) by IIAA assay (Y) with those by electrophoretic analysis (X) for 51 sera obtained from 20 patients.

Group 1 (+): 10 patients (24 samples); Group 2 (O): five patients (13 samples); Group 3 (D): five patients (14 samples)

Fig. 3. Densitometric tracings from selected serum samples

For each sample untreated serum and an equivalent volume of supernate from IIAA-treated aliquots were analyzed simultaneously by agarose electrophoresis. Amylase activity in untreated sera is denoted by the solid line outline, the amylase activity in the IIAA-treated supernate by the shaded areas. Total amylase (U/L): Sample A, 297; B, 327; C, 304; D, 767. The mobility of the salivary amylase marker is shown by the solid bar in each frame.
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5 U/L); (total nonparametric tation total deficiency. use 90% method and day-to-day within-run IAA Lanes I1AA Fig. 4. Electrophoretic analysis of serum and urine samples treated with IIAA suspension Lanes 1 and 8, purified salivary amylase; lanes 2 and 3, a normal serum sample (total amylase = 50 U/L); lanes 4 and 5, normal urine 1 (total amylase = 224 U/L); lanes 6 and 7, normal urine 2 (total amylase = 82 U/L). Samples in lanes 3, 5, and 7 were treated with the antibody suspension.

Discussion

The present study establishes that indirect immobilization of murine anti-salivary amylase antibody onto particles of polyvinylidene fluoride can be used to prepare a suspension that is useful for measuring pancreatic amylase in serum. This assay's properties—precision, removal of salivary amylase, and correlation with electrophoretic results—are similar to those of our earlier assay (18).

When compared with other pancreatic amylase methods (16, 21, 29–34), the IIAA provides excellent performance. Its within-run CV is among the lowest reported whereas its day-to-day CV is the lowest (1–2%). When considered as a group, the three precipitation immunoassays [IIAA, DIAA, and the bacterial immobilized amylase antibody (IIIA)] remove salivary amylase much more effectively (>99%) than the inhibition immunoassay for amylase (only 90% selectivity) (17). Moreover, the presence of this residual salivary amylase activity in the latter assay requires the use of a rather elaborate calculation to compensate for this deficiency.

Because of the nongaussian distribution of the data for total amylase, pancreatic amylase, and % pancreatic fraction (Table 3), the reference intervals were determined by nonparametric analyses. We also observed nongaussian distributions for the reference intervals derived with the other two substrates. Clearly, reference intervals for % pancreatic amylase activity will depend on the substrate used. Compared with pancreatic amylase, salivary amylase displays a preference for short-chain oligosaccharides (35). We expected longer oligosaccharides to yield more activity with pancreatic amylase (and correspondingly increase the % pancreatic fraction), and indeed we observed this for the p-nitrophenylheptaoside substrate.

Last year we reported (36) that this antibody appeared to react with macroamylases in an unbiased fashion; i.e., the mean % pancreatic amylase in macroamylasemic sera was not significantly different from the mean % pancreatic amylase in normal sera. Analyses of macroamylases by this monoclonal antibody thus would not be expected to yield misleading results, although further studies are needed to establish this response.

From the data in Figure 2, we conclude that the method of immobilization has little or no effect on the immunochemical reaction of this antibody (compare the results shown in Figure 2 with those of Figure 2 in reference 16). In addition, examination of individual data from subpopulations of patients shows no apparent analytical bias for patients diagnosed with or without pancreatitis (Figure 2). In such patients, isoforms of pancreatic amylase may appear, with pI values similar to those of salivary amylase. These regression results from Figure 2 are similar to our earlier observations (18).

The results obtained in this study by agarose gel electrophoresis merit some additional comment. Fixed-voltage electrophoresis in agarose is commonly used to separate amylase isoenzymes and isoforms (3, 29, 30). A key advantage of electrophoresis is its ability to provide moderate resolution of the principal amylase isoenzymes and some isoforms—one of which, pancreatic amylase form P3, reportedly has potential diagnostic utility for pancreatitis (31, 37). However, the method involves lengthy processing time (e.g., 3–4 h) and has poor analytical sensitivity. Moreover, we found, in the present studies, that agarose gel electrophoresis may not completely resolve all of the amylase isoforms into separate, unique bands.

In fact, our results suggest that the electrophoretic mobilities of some pancreatic isoforms of amylase in serum overlap those of salivary isoforms. Serum from which salivary amylases had been removed immunochemically continued to show amylase activities that migrated in the "salivary" region of the electrophoretic plate (Figure 3). This remaining amylase activity cannot be considered salivary, because the anti-salivary antibody completely removes all isoforms of salivary as well as ovarian amylase (14). Gerber et al. (17) found similar results, using a different (inhibiting) antibody and isoelectric focusing rather than constant-voltage electrophoresis. These findings are consistent with the overlapping isoelectric points of salivary and pancreatic isoforms (see, e.g., reference 26). Thus it appears that identification of amylase isoenzymes, based on comparison of their mobilities to the mobility of purified marker(s), may lead to incorrect classification of the isoenzymes. Not only are these acidic pancreatic amylase isoforms obscured from identification, they are incorrectly quantified as salivary amylases. The fact that results by the electrophoretic method correlated so well with those by other methods suggests that the misidentification problem may be of only minor significance for most samples. However, if quantification of individual amylase isoforms is desired (e.g., P3), then reli-

Table 3. Nonparametric Reference Intervals for Pancreatic Amylase

<table>
<thead>
<tr>
<th>Pancreatic Amylase</th>
<th>Boehringer-Mannheim, p-nitrophenylheptaoside</th>
<th>DuPont, maltopentaose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic amylase, U/L</td>
<td>9–58</td>
<td>19–89</td>
</tr>
<tr>
<td>Pancreatic fraction, %</td>
<td>16–78</td>
<td>27–73</td>
</tr>
<tr>
<td>n = 140 subjects.</td>
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</table>
able results may not be possible with some electrophoretic methods (e.g., fixed voltage in agarose gels). Pre-treating serum with anti-salivary amylase before electrophoresis may improve this situation.

Inspection of data obtained from analysis of the urine samples suggests that the IAA assay can also provide an accurate determination of pancreatic amylase in urine. We conclude that this monoclonal antibody method is uninfluenced by usual urinary constituents and yields analytical performance equivalent to that demonstrated for serum.

The present studies provide initial information about the clinical performance of this assay. In all patients studied who had clinically diagnosed acute pancreatitis, the measured pancreatic amylase in serum exceeded the upper limit of the normal reference interval, as did the pancreatic fraction (in percent) of the total amylase in serum. The latter finding appears important because of previous evidence that serum pancreatic (and total) amylase (U/L) may be increased nonspecifically when amylase clearance is impaired (38). Further studies are needed to document the clinical sensitivity and specificity of the IAA assay in other groups of patients.

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References