Improved DEAE-Sephadex Column Chromatography in Measuring Amylase in Serous Ovarian Neoplasms, and Results for 13 Cases

Jack J. Zakowski and David E. Bruns

We describe a column-chromatographic method for measuring amylase activity in cyst fluids of serous ovarian tumors. Using this technique, we confirm and extend the previous report of an "acidic" amylase in serous ovarian tumor cyst fluids. This form of the enzyme was eluted from DEAE-Sephadex mini-columns with a high-salt buffer. It accounted for 45 to 100% of the amylase in 13 cyst fluids. This "high-salt" amylase was also present in the tumor tissues. The acidic nature of the amylase does not appear to be due to sialic acid residues, because the chromatographic behavior of the amylase is unaffected by treatment with neuraminidase. We conclude that the "acidic" amylase reported previously in two serous ovarian tumors is a constant feature of these tumors and that it is distinct from the sialylated tumor amylase described by others.

Additional Keyphrases: cancer "marker" • activity in serous cyst fluid compared with that in mucinous cyst fluid and with tumor tissue • enzyme activity • pancreatic, salivary isoenzymes compared • diagnostic aid

We recently reported that cyst fluids from serous ovarian tumors contain large amounts of amylase activity (1). The finding that increased amylase is uniquely associated with the serous type of ovarian tumor (1) is important, because this is the most common type of ovarian cancer (2). The amylase found in the cyst fluids from two patients was found (1) to be distinguishable from pancreatic and salivary amylases by virtue of its delayed elution on DEAE-Sephadex chromatography. However, the method used in those studies required the use of two chromatographic columns and relatively large sample volumes and did not lend itself to studying larger numbers of specimens.

Here we describe the use of a single mini-column of DEAE-Sephadex to quickly measure the amylase isoenzyme in serous ovarian tumors. We examined cyst fluids from 13 additional serous ovarian tumors and found that all of them contained the distinctive isoenzyme of amylase that is eluted in the high-salt buffer ("high-salt" amylase).

Methods

DEAE-Sephadex Mini-Column Chromatography

DEAE-Sephadex (Sigma Chemical Co., St. Louis, MO 63178) was reconstituted in a mixture containing, per liter, 500 mmol of NaCl and 30 mmol of Tris (pH 7.15) and then extensively washed in 30 mmol/L Tris, pH 7.15. Polypropylene mini-columns (0.8 x 7 cm; Bio-Rad Laboratories, Richmond, CA 94804) were filled to a 3-cm bed height with gel (2 mL final bed volume) and washed with three to five bed volumes of the low-salt buffer (30 mmol/L Tris, pH 7.15). Samples of 10 to 250 µL were applied to the columns and washed with 10 successive 0.6-mL aliquots of low-salt buffer. High-salt amylase was then eluted with 10 0.6-mL aliquots of a 250 mmol/L NaCl, 30 mmol/L Tris mixture, pH 7.15. Each aliquot of added buffer was collected as a separate fraction. All chromatography was done at room temperature.

Amylase Assay

We assayed for amylase essentially as described elsewhere (3) in a Rotochem IIa/36 Centrifugal Analyzer (American Instruments Co., Silver Spring, MD 20910) by use of a coupled enzymatic assay with maltotetraose as substrate (Amylase-DS Reagent; Beckman Microbics, Carlsbad, CA 92008). The reference range for serum amylase with this assay is 20-80 U/L. For assays of effluent fractions, we increased the sensitivity of the assay by increasing the sample volume to 0.2 mL and decreasing the reagent volume to 0.3 mL. Amylase-DS reagent was reconstituted with an appropriate volume of diluent so as to give the correct final concentrations of reactants after mixing. After a 3-min lag period, the absorbance at 340 nm was measured every 30 s for 4 min, to calculate amylase activity. One unit is defined as the amylase activity that produces 1 µmol of NADH per minute under the conditions of the assay.

Sample Preparation

Serum samples collected by the usual venipuncture and centrifugation procedures were used without further preparation. Ovarian cyst fluids, obtained postoperatively and centrifuged to remove debris, contained 20 to 150 g of total protein per liter. Tissue specimens were obtained postoperatively, homogenized, centrifuged, and the supernates used without further preparation.

Enzymatic Modification of Amylase

Neuraminidase (EC 3.2.1.18; type X, from Clostridium perfringens) was obtained from Sigma Chemical Co. Samples of amylase were mixed with an equal volume of sodium acetate buffer (0.15 mol/L, pH 5.5) containing neuraminidase (final concentration = 15.2 kU/L). Controls were run identically except that enzyme was omitted. Samples were incubated at 37 °C for 26 h, then assayed for residual total amylase and percentage high-salt amylase.

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Results

DEAE-Sephadex Chromatography

Approximately 90% of the amylase activity in normal serum was eluted from mini-columns of DEAE-Sephadex with only the low-salt column buffer (Figure 1A). By contrast, most ovarian tumor amylase became bound to the mini-columns and was eluted only with the buffer of high salt concentration (Figure 1B). At the pH (7.15) used here, the salivary and pancreatic isoenzymes of amylase are eluted together in the void volume. Samples of saliva, serum from patients with acute pancreatitis, and normal urine all showed the same elution profile as that for normal serum (data not shown). A matrix effect is not involved in producing the observed difference between cyst fluid and other samples, because mixing one part of ovarian cyst fluid (as in Figure 1B) with 100 parts of normal serum (as in Figure 1A) had no effect on the elution behavior of either the low-salt or high-salt fractions (Figure 1C). A similar mixing experiment with normal urine in place of serum gave identical results (data not shown). In all these mixing experiments the analytical recovery of amylase in each peak was within 5% of that predicted from the initial sample activities.

The DEAE-Sephadex chromatography must be completed within 60 to 90 min after the mini-column has been equilibrated, because long exposure of the Sephadex to the buffer, particularly the high-salt buffer, results in an artifact that produces apparent amylase activity in this assay procedure. This artifact evidently is a carbohydrate breakdown product of the Sephadex, because columns run without sample also produced this interference and the interfering substance did not produce a linear, steadily increasing absorbance in the amylase assay but was characterized by a rapid increase in absorbance during the first 3 to 4 min, quickly followed by a leveling off of absorbance.

Some Analytical Variables

Linearity. An aliquot of ovarian cyst fluid was diluted 100-fold with normal serum and increasing volumes of this mixture were assayed by the present procedure. The resulting curve for the data was linear to at least 900 U of amylase per liter (Figure 2). The relative amounts of low-salt and high-salt amylase remained constant over the range investigated.

Precision. Precision of the procedure was determined, both for within-run and day-to-day values. High coefficients of variation (10% for within-run and 48% for day-to-day) were seen for the low percentages (<13%) of high-salt amylases found in normal sera. Much lower CVs were noted for the higher percentages of high-salt amylase found in ovarian cyst fluids (0.5% for within-run and 3.2% for day-to-day).

Amylase in Patients’ Samples

Cyst fluids from a series of 13 serous ovarian tumors were collected, analyzed by the present procedure, and the percentage of high-salt amylase was calculated (Table 1). All cyst

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Total amylase, U/L</th>
<th>% high-salt amylase</th>
<th>Diagnosis</th>
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<tr>
<td>1</td>
<td>142</td>
<td>100</td>
<td>Serous adenoma + amylidosis</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>98</td>
<td>Serous adenoma</td>
</tr>
<tr>
<td>3</td>
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<td>86</td>
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</tr>
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<td>100</td>
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</tr>
<tr>
<td>5</td>
<td>415</td>
<td>55</td>
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<td>6</td>
<td>612</td>
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<td>2700</td>
<td>45</td>
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</tr>
<tr>
<td>13</td>
<td>8025</td>
<td>89</td>
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fluids contained amylase activities exceeding the reference range for serum (20–80 U/L) and had high percentages (45 to 100%) of high-salt amylase. Patient no. 8 had bilateral ovarian tumors, one serous and the other mucinous. As expected (1), the serous tumor showed a high total amylase activity (820 U/L, 100% high-salt) while the mucinous did not (15 U/L).

We investigated the amylase from the tumor tissue of two of the patients. In patient no. 4 (Table 1) the cyst fluid amylase was all in the high-salt fraction, while only 39% of the amylase activity in the corresponding homogenized tumor tissue was in the high-salt fraction. Similarly, in patient no. 1, whose cyst fluid contained 53% high-salt amylase, 35% of the total amylase in the homogenized tumor tissue was in the high-salt fraction.

Effect of Neuraminidase Treatment

The distinctive characteristics of tumor amylases could reflect their sialylation. We therefore incubated high-salt amylase with neuraminidase to remove sialic acid residues, and then re-chromatographed the amylase. This treatment of high-salt amylase from ovarian cyst fluid did not convert it to the low-salt form. Evidently the increased negative charge on the high-salt amylase is not ascribable to the presence of neuraminidase-removable sialic acid residues. There was no significant loss of enzymatic activity after incubation, with or without neuraminidase.

Discussion

There have been numerous reports of hyperamylasemia associated with tumors of several organs (4–9), including cancer of the ovaries (1, 10–12). We found high amylase activity in all of 13 serous ovarian cyst fluids (range = 140–8025 U/L) as we reported previously for a smaller series (1). Amylase activity was low in several mucinous ovarian cyst fluids (range = 2–30 U/L) as our previous study led us to expect (1). Amylase activities tended to be greater in borderline and malignant tumors than in benign tumors (median activities of 2512 U/L and 649 U/L, respectively; Table 1). Amylase activity did not correlate with cyst volume or with the age or race of the patient (data not shown).

The nature of the ovarian tumor amylase has been controversial. Several authors (13, 14) could not distinguish the tumor amylase from the saliva type. Other authors have demonstrated ovarian tumor amylases with distinctive mobilities on electrophoresis on cellulose acetate (15) and agarose gel (11). Van Kley et al. (1, in two further cases, showed an isoenzyme of amylase that could be separated chromatographically and that also showed a more negative charge on isoelectric focusing, with a pI of 5.25. Warshaw and Lee (16) reported an amylase isoenzyme with a similar pI (5.23) in the serum of an additional patient with an ovarian tumor. Tak-euchi et al. (4) studied the amylase from one ovarian tumor, finding it immunochemically similar to the pancreatic and salivary isoenzymes. We confirmed this similarity in a series of ovarian tumors, using an immunoperoxidase technique (17). These previous procedures used to study the presence of an ovarian tumor amylase isoenzyme have drawbacks; they are either only semiquantitative, as in electrophoresis and histochemistry, or long and laborious, as in the column-chromatographic separations and isoelectric focusing.

The present procedure is both quantitative and technically simpler than the methods used previously. Samples can be applied, without preparation, directly to this column and chromatographed in about 1 h. Normal serum was seen to contain only small amounts of high-salt amylase. Samples of ovarian cyst fluids showed strikingly higher percentages of high-salt isoenzyme. In our series of 13 cyst fluids from serous ovarian tumors the percentage of high-salt amylase isoenzyme ranged from 45 to 100% of the total amylase. Interestingly, tissue samples from two of the tumors contained lower percentages of this isoenzyme than did the associated cyst fluids, but still substantially higher total activity than normal serum. These intermediate results for tumor tissue may reflect the presence of normal serum amylases from the blood in these tumors. Alternatively, tumor amylase may be synthesized as a “low-salt” enzyme that undergoes subsequent modification to a more acidic form. The availability of a rapid technique for measuring the “high-salt” enzyme may prove important in designing experiments to examine the latter possibility.

As evidenced by its increased binding to the DEAE-Sephadex in our studies and that of Van Kley et al. (1), who also demonstrated a lower isoelectric point for the tumor amylase, the high-salt amylase has a greater negative charge than do the pancreatic or salivary enzymes. The presence of sialic acid residues has been proposed (18) as an explanation for the increased negative charge of tumor amylases, but we found that incubation of ovarian cyst fluid with neuraminidase did not cause this isoenzyme to be eluted with the low-salt buffer in our mini-column procedure, indicating that this proposal is incorrect, at least with respect to readily removable sialic acid residues.

Measurement of this form of amylase in serum or urine may provide a useful marker for serous ovarian tumors. Further studies are required to establish its relationship to other amylases, such as those found in pancreatic pseudocysts (19). We expect the present assay to be a useful tool during the purification and characterizations of serous ovarian tumor amylase that are currently underway in our laboratory.

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Four Methods for Determining Uric Acid Compared with a Candidate Reference Method

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Uric acid as measured in serum by three different uricase (EC 1.7.3.3) methods (aca, Ektachem, and SMAC) and by the SMAC method with phosphotungstic acid was compared with a candidate Reference Method for uric acid. Serum specimens from 83 patients (uric acid concentrations, 19 to 141 mg/L) were analyzed by all five methods. Results were compared by using linear regression analysis, and the mean deviation between results by the candidate Reference Method and the four other methods was calculated. Compared with the candidate Reference Method, the aca method gave the smallest deviation from zero for the intercept and the smallest mean difference, and the SMAC phosphotungstic acid method showed a slope closest to unity. The SMAC uricase method had the largest intercept and greatest deviation of the slope from unity.

Additional Keyphrases: enzimic methods • discrete analysis • continuous-flow analysis • multilayer film analysis

Two widely accepted methods are used to determine uric acid in serum. In the older method uric acid reduces colorless phosphotungstic acid (PTA)1 to tungsten blue, which usually is measured by its absorbance at 700 nm. The newer method involves enzymic conversion of uric acid and oxygen to allantoin and hydrogen peroxide by the enzyme uricase (urate oxidase, EC 1.7.3.3; urate:oxygen oxidoreductase). Several uricase methods are available, differing in their indicator systems. Because common biological reducing substances such as glutathione, ascorbic acid, and several antibiotics cause a positive interference in the PTA determination of uric acid, it is generally accepted that there is a constant bias between the two methods, with the uricase result being 1 to 10 mg/L lower than the PTA result (1-3). The initial uric acid method available for the Sequential Multiple Analyzer Computerized (SMAC; Technicon Instruments Corp., Tarrytown, NY 10591) was the PTA method. Recently, Technicon introduced a uricase method for determination of uric acid with the SMAC. Our laboratory also can determine uric acid concentration with the Automatic Clinical Analyzer (aca; Du Pont Instruments, Wilmington, DE 19898) and the Ektachem (Eastman Kodak Co., Rochester, NY 14650), in both of which a uricase method is used. To select the optimum method for the SMAC and to determine comparability among the four methods mentioned, we compared results by all four methods with those by a candidate Reference Method—a uricase method involving equilibrium kinetics and measurement of ultraviolet absorption (4,5).

Materials and Methods

Samples. Blood specimens from patients in the Clinical Center, submitted to the Clinical Chemistry Service for uric acid measurement, are determined with the SMAC PTA method. We selected specimens for this study by reviewing the uric acid results so obtained, selecting specimens that contained 3 mL or more of serum and that were distributed uniformly over the range of concentration for uric acid. Thus, we tried to obtain an equal number of specimens below the lower limit of the reference interval, within the reference interval, and above the reference interval. The serum specimens, stored at -20 °C, were thawed the same day that the analyses were performed by the five methods. A human serum pool obtained from normal volunteers and stored at -70 °C was also assayed each day of the study by the five methods.

Apparatus. The spectrophotometer used with the candidate Reference Method was equipped with a 37 °C temperature control and ultraviolet source (Model 25; Beckman Instruments, Inc., Carlsbad, CA 92008). The SMAC, aca, and Ektachem were calibrated and used according to the manufacturers’ instructions.

Candidate Reference Method. The candidate Reference Method was used as described (4,5). Based on the ultraviolet uricase equilibrium method of Remp (6), this method requires both a test and a blank: Incubate 0.5 mL of specimen with 2.5