Determinations of Amylase Isoenzymes in Serum by Use of a Selective Inhibitor

William Y. Huang and Norbert W. Tietz

A simple, rapid screening procedure for determining the relative amounts of pancreatic (P)- and salivary (S)-type amylase in serum is presented, involving incorporation of a selective inhibitor (from wheat-germ) in commercially available BMD Single-Vial Amylase and Beckman Enzymatic Amylase-DS procedures for manual and automated isoamylase measurements. Optimal concentrations of inhibitor inhibit the S-type amylase fraction by 87–88%. In contrast, the pancreatic fraction is inhibited by only 19% in either the manual or automated methods. The degree of inhibition is constant for amylase activities up to at least 520 U/L. Use of the ratio (P-amylase/total amylase activity) × 100 helps differentiate between hyperamylasemia caused by S-type or P-type amylase. In preliminary studies, patients with pancreatitis showed a ratio > 70%.

Additional Keyphrases: enzyme activity • pancreatitis • hyperamylasemia • reference interval • screening

Serum α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) is heterogeneous and exists in different isoenzyme forms. Salivary type (S-type) and pancreatic type (P-type) amylases are apparently the products of two closely-linked loci on chromosome 1 that code for amylase isoenzymes (1). Although several different tissues synthesize the S-type amylase (e.g., salivary, lacrimal, and sweat glands; genital tissues; lung; bronchogenic and ovarian tumors; lactating mammary glands; leukocytes and thrombocytes), the P-type amylase is essentially tissue-specific, semen being the only other known source (1, 2).

Because amylase is present in a variety of tissues, hyperamylasemia not only may occur in pancreatic disorders, but also in mumps, intestinal obstruction and other intestinal disorders, and in cases of tumors of the ovaries and lungs. It also has been observed postoperatively as a concomitant of urinary retention, in diabetic ketoadiposis, and in macroamylasemia (2, 3).

Measurement of amylase isoenzymes can therefore serve to confirm pancreatic disease or aid in the diagnosis of hyperamylasemia of unknown origin by excluding pancreatic involvement. Furthermore, it may be of decided value in evaluating post-surgical hyperamylasemia (4).

Present methods for separating amylase isoenzymes—such as ion-exchange chromatography, electrophoresis, and isoelectric focusing (5)—are relatively time-consuming, and we and others (6) find that there is some overlap in electrophoretic migration rate between the slowest-moving S-fraction and the fastest-moving P-fraction.

For clinical (and especially emergency) applications, a simple, rapid, and reliable method for isoamylase determinations is most desirable. Such a method was made possible by the isolation of an α-amylase inhibitor from wheat germ (7, 8), which is claimed to be 100-fold more specific for human S-type amylase than for P-type amylase. This inhibitor was subsequently incorporated into an amylase procedure in which insoluble dye-coupled starch was the substrate (7). However, this method is relatively labor intensive, requiring a 25-min preincubation of sample with inhibitor, a 15-min incubation of substrate with sample, and subsequent centrifugation or filtration before photometric measurement; moreover, turbidity that persists after centrifugation frequently interferes with the photometry.

We have adapted the original method by O'Donnell and McGeeney (7) to a widely used enzyme-coupled determination of total amylase activity, involving maltotetraose as a substrate. This method is relatively simple and rapid, has a wide range of linearity, and can easily be adapted to a centrifugal analyzer.

Material and Methods

Apparatus. For the manual procedure described below we used a spectrophotometer (Model 28; Beckman Instruments, Inc., Fullerton, CA 92634). The automated adaptation involved use of a centrifugal analyzer (Rotochem II-A; Travelsol Laboratories, Hyland Diagnostic Instruments Division, Deerfield, IL 60015).

Reagents. The amylase inhibitor, a protein (Mr 21,000) purified from wheat germ (7), was kindly contributed by K. F. McGeeney (Dublin, Ireland). By disc electrophoresis its major band has a mobility 0.2 that of bromphenol blue. The purified preparation must be stored frozen at −70 °C. We prepared a concentrated stock solution by diluting c=720 μg of inhibitor with 1 mL of inhibitor diluent containing phosphate buffer (50 mmol/L, pH 6.9), calcium chloride (0.5 mmol/L), and sodium chloride (50 mmol/L), following the method of O'Donnell et al. (7, 8). We stored 20 50-μL aliquots of this stock solution at −70 °C. Repeated freezing and thawing reduces inhibitor activity, so, when needed, an aliquot was removed from the freezer and diluted with buffer, as described later. These thawed solutions are stable for five days, if refrigerated at 4–8 °C or kept in ice water.

The amylase reagents used in the study were the BMD Single Vial Amylase Reagent kit (Boehringer-Mannheim Diagnostics, Inc., Houston, TX 77036; cat. no. 145000, UV-method), and the Beckman Enzymatic Amylase-DS Reagent kit (Beckman Instruments, Inc., Microbes Operation, Carlsbad, CA 92008). Both kits are based on the same methodological principle and were purchased directly from the manufacturers.

Samples. Pancreatic extracts were prepared by homogenizing pancreatic tissue in a blender, then adding about five times the volume of bovine serum albumin solution (50 g/L), mixing, and centrifuging at 1000 x g for 10 min. We diluted the supernate with aca Enzyme Diluent2 (DuPont Co., Wilmington, DE 19898) to obtain solutions with the desired amylase activity.

---

2 The DuPont aca Enzyme Diluent has the following composition: Na, 105–120 mmol/L; K, 4.0–5.0 mmol/L; Cl, 98–112 mmol/L; Mg, 20–30 mg/L; Ca, 80–120 mg/L; PO4 (as phosphorus), 30–50 mg/L; SO4 (as sulfur), 10–20 mg/L; ZnCl2, 5 mg/L; purified bovine albumin, 60–70 g/L; pH, 7.1 ± 0.3.

Received Feb. 8, 1982; accepted April 23, 1982.

Division of Clinical Chemistry, Department of Pathology, University of Kentucky Medical Center, Lexington, KY 40536.

1 Address correspondence to this author.
Saliva collected from laboratory personnel was centrifuged at 1000 x g for 10 min. The clear supernate was then diluted with DuPont Enzyme Diluent as required.

**Manual procedure.** Place 50 μL of sample into a 10 x 75 mm Pyrex test tube and add 10 μL of inhibitor solution (diluted 30-, 60-, 120-, or 240-fold, respectively), resulting in inhibitor concentrations of ≈0.24, 0.12, 0.06, and 0.03 μg per 50-μL sample, respectively. The total activity in each sample is measured by replacing inhibitor with inhibitor diluent.

Mix and incubate for 5 min at 37 °C and add 1.0 mL of BMD or Beckman amylase reagent, prewarmed to 37 °C. Transfer the solution to a spectrophotometer microcuvette (10-mm light path), place this in the spectrophotometer cuvette compartment (37 °C), and monitor the change in absorbance (ΔA) at 340 nm, preferably with a recorder. Calculate ΔA/min from the linear portion of the reaction rate curve, generally from 5 to 8 min after initiation of the reaction.

After approximately 10 min, there is a gradual increase in the reaction rate, probably from dissociation of the enzyme–inhibitor complex, possibly as a result of the formation of a substrate–inhibitor complex (9).

Calculate the results as follows:

\[
\frac{(\Delta A/\text{min}) \times \text{total assay vol (mL)} \times 1000}{\text{millimolar absorptivity} \times \text{specimen vol (mL)}} = \frac{\text{U/L}}{
\frac{(\Delta A/\text{min}) \times 1.06 \text{ mL} \times 1000}{6.22 \times 0.05 \text{ mL}}} = \frac{(\Delta A/\text{min}) \times 3408}{\text{U/L}}
\]

where 6.22 is the millimolar absorptivity for NADH as measured in a cuvette with a 10-mm light path, and 1000 converts millimoles to micromoles.

**Automated procedure.** The procedure can be readily adapted to a centrifugal analyzer by using 20-μL samples, flushing with 80 μL of distilled water (with and without 20 μL of inhibitor), and adding 400 μL of amylase reagent. After a 5-min preincubation, the reaction is initiated and readings are taken after a 5-min lag time.

**Results and Discussion**

Figure 1 illustrates the effect of various inhibitor concentrations on the activity of pancreatic and salivary amylase. The inhibitor concentration of 0.06 μg per 50–μL sample is best for the determination of the relative percentage of the P- and S-amylase isoenzymes, because ≈81% of the P-isoenzyme activity (but only 13% of the S-isoenzyme activity) is retained. A lower inhibitor concentration (0.03 μg per 50-μL sample) inadequately inhibits the S-type isoamylase, ≈22% of its activity remaining.

The inhibitor concentration we selected is only about 12% of that proposed by O'Donnell et al. (8), a change made possible by our more favorable reaction conditions (smaller reaction volume, shorter incubation interval for the enzyme reaction, and use of a different substrate).

Prolonging the preincubation for the sample/inhibitor mixture to 10 min did not significantly change inhibition patterns of the two isoenzymes.

The most appropriate dilution of the inhibitor must be determined for each lot of inhibitor, because of changes in its potency as a result of lot-to-lot variability or loss of potency as a result of extended storage.

The amount of inhibitor we recommend for the assay, 0.06 μg per 50–μL sample, is adequate for enzyme activities (of untreated sample) to at least 520 U/L, which is more than fourfold the upper reference limit of 120 U/L (Figure 1). To avoid unacceptably high initial absorbance readings one must dilute samples with greater amylase activities, either before or after incubation of the sample with the inhibitor.

The sequence of addition of enzyme and substrate is critical for maximum isoenzyme inhibition. The inhibitor must first react with the enzyme to form an enzyme–inhibitor complex, before the substrate is added. Adding the inhibitor directly to the substrate, followed by addition of the enzyme, leads to less inhibition, possibly because a substrate–inhibitor complex may form (9).

**Standard curve.** A standard curve can be prepared by mixing various proportions of saliva and pancreatic extract, and determining the respective activity of each in the presence and absence of amylase inhibitor. The mixture of P- and S-type amylase is best represented by the ratio between P-isoenzyme and total activity (T) (=P + S) activity x 100. Figure 2 shows a plot of remaining total activity vs (P/T) x 100. The demonstrated linear contrast with the results of O'Donnell et al. (8), who found a non-linear relationship when they used the "Phadebas" method (Pharmacia AB, Uppsala, Sweden) and the same inhibitor. This may have been the result of either a different affinity of inhibitor to substrate, or the longer reaction period, which would allow a greater degree of dissociation of the enzyme–inhibitor complex, as discussed above.

**Precision of the proposed method.** The procedure without inhibitor, as determined on 20 patients' sera with normal and increased total amylase activity, measured in a single run, gave
results of 89 U/L (SD 1.4) and 302 U/L (SD 4.8), with CVs of 1.6% for both. Corresponding values obtained with the inhibitor present were 61 U/L (SD 0.9) and 230 U/L (SD 4.4), with respective CVs of 1.5% and 1.9%. Run-to-run variability (n = 14) was determined by repeated analyses with “Monitrol I” control material (Dade Division, American Hospital Supply Corp., Miami, FL 33152). The mean values for this control, with and without inhibitor, were 106 U/L (SD 1.5) and 26 U/L (SD 1.9), with CVs of 2.2% and 1.4%, respectively. The corresponding values for “Monitrol II” were 459 U/L (SD 9.9) and 75 U/L (SD 6.6), with CVs of 8.8% and 7.3%, respectively.

Clinical utility. Some initial data obtained with this method for serum from patients with hyperamylasemia are shown in Table 1. In each of the five examples of pancreatitis listed, the percent of P-type isoamylase (73-96%) exceeded the normal range for this ratio, which is 27-70% (mean 48%). In the two examples of carcinoma of the lung, the (P/T) × 100 ratio was substantially lower (15 and 20%) than the reference interval. A serum specimen collected from a patient just after oral surgery showed a ratio of 29%.

The P-type amylase activity in the serum of 27 healthy individuals with a total amylase activity of 34-124 U/L (mean 63) and a (P/T) × 100 ratio of 27-70% (mean 48%) was found to be 16-45 U/L (mean 29) and for the S-type amylase 11-87 U/L (mean 34).

The observed activities for P- and S-type amylase differ slightly from those observed by other authors. Gillard (10), using quantitative polyacrylamide gel electrophoresis, found an average of 43% (SD 8%) of P-type amylase in sera of healthy persons, while Legaz and Kenny (11), using cellulose acetate electrophoresis, reported 55% (SD 12%) of P-type amylase. These differences must be attributed to differences in methodology. The method of selected inhibition, as already pointed out by Berk et al. (12), is somewhat less reliable for samples with extremely low (≤9%) or extremely high percentages (≥89%) of P-type amylase. However, this does not decrease the clinical utility of this simple, fast screening method.

An extensive evaluation of the clinical utility of isoamylase determinations in serum by selective inhibition is currently under way and will be reported in a separate communication. In this additional study we will also compare this method with other new or established procedures.

Adaptation to other amy lase procedures. We also conducted preliminary experiments to evaluate the suitability of selective inhibition by the wheat-germ inhibitor, utilizing the acxa method and a newly developed amylase procedure (Boehringer Mannheim Diagnostics, Inc.) in which 4-nitrophenylmaltotetraose is the substrate. Both of these methods can be adapted for P-type amylase determinations, but the optimum ratio of inhibitor to sample size differs for these methods and needs to be established in each respective laboratory.

References