Catalytic Concentrations of Amylase Isoenzymes: An Assay with Wheat-Germ Inhibitor and 4-Nitrophenylmaltotetraoside plus 4-Nitrophenylmaltohexaoside as Substrate

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We coupled a kinetic procedure to a selective inhibiting method for determining amylase isoenzymes in biological samples, using 4-nitrophenylmaltotetraoside plus 4-nitrophenylmaltohexaoside as substrate and a wheat-germ selective inhibitor with the Gillard S-III spectrophotometer. On plotting remaining amylase activities/total amylase activities (R/T) vs pancreatic amylase activities/salivary amylase activities (P/S) ratios, we found the curve to be linear for P/S ratios from 0.2 to 5. The inhibition rate of amylase inhibitor was constant in solutions having total amylase activities between 20 and (at least) 900 U/L. CVs were 3.1 to 7.1% for pancreatic amylase and 2.0 to 12.9% for salivary amylase in serum. Correlation with the Phadebas method was excellent (r = 0.99) for both pancreatic and salivary amylase. We also automated this procedure in an Hitachi 705 analyzer and correlated the results (r = 0.99) with those by our manual method.

Additional Keyphrases: kinetic enzyme assay · pancreatic disease · hyperamylasemia · reference interval · age-related effect

Two major sources of α-amylase (EC 3.2.1.1) in humans are the salivary glands and the pancreas (1). The multiple isoenzymes demonstrated in many fluids and tissue extracts are thought to be heterogeneous products of the two gene loci, Amy 1 (salivary type amylase) and Amy 2 (pancreatic type amylase) (2). Isoamylase analysis is useful in the differential diagnosis of hyperamylasemia. The existence of isoenzymes of amylase has been established by electrophoretic, isoelectric focusing, and chromatographic techniques (3). However, these procedures are either technically too complex or time consuming for routine clinical laboratory use. A commercial kit involving an amylase inhibitor, following the principle of the method of O’Donnell et al. (4), made available by Pharmacia, has made prompt assay of isoamylase possible. Recently, several synthetic substrates for amylase assay with kinetic methods have been reported, involving a coupled enzyme assay (5, 6). We have adapted the original method of O’Donnell et al. to a widely used enzyme-coupled determination of total amylase activity. The substrate we have chosen (NPG 5/6) and NPG-7 have defined chemical structures, and they are used in a defined concentration (6). On the other hand, NPG-4 is the main product (about 60%) of the amylase reaction with the NPG-7 substrate. This product is split only to a very slight extent by the α-glucosidase (which is used as an indicator reaction in both NPG-7 and NPG 5/6 methods), and it accumulates in

1 Nonstandard abbreviations: NPG 5/6, 4-nitrophenylmaltotetraoside plus 4-nitrophenylmaltohexaoside; NPG-7, 4-nitrophenylmaltobetaoside; NPG-4, 4-nitrophenylmaltotetraoside; P, S-amylase, pancreatic and salivary amylase isoenzymes, respectively.
the reaction mixture. Because NPG-4 is an inhibitor of the amylase reaction (6) and, moreover, does not yield free p-nitrophenol by the α-glucosidase activity, its accumulation reduces the sensitivity of NPG-7 test. NPG 5/6 substrate produces NPG-4 only to a very small extent, not more than 5% of the total product (6). The procedure with NPG 5/6 thus is relatively simple, fast, and can be adapted to an automated kinetic system.

Materials and Methods

Apparatus. For the manual method we used a Model S-III spectrophotometer (Gilford Instruments Laboratories Inc., Oberlin, OH 44074) with a Compucorp 5000 supplier. An Hitachi 705 discrete automated analyzer (Boehringer Mannheim Diagnostics, Mannheim, F.R.G.) was used for the automated method.

Reagents. Amylase inhibitors were obtained from Pharmacia Diagnostics, Upseal, Sweden, as were standards of human pancreatic and salivary amylase. The substrate NPG 5/6 we used was from Behringwerke AG, Marburg, F.R.G.

Procedure. For the assay we used 20 μL of sample, adding 600 μL of distilled water or inhibitor to obtain total or remaining amylase activities, respectively. We added 1000 μL of amylase reagent to the mixture after a 25- to 30-min preincubation. Readings were taken 5 min after the reaction was initiated to determine the catalytic activity. The incubation temperature was fixed at 37 °C and the wavelength at 405 nm.

In studying how to automate this procedure we used a serum having a high amylase activity, 900 U/L, from a patient with an ovarian carcinoma, and a salivary standard having an activity of 900 U/L. We even added different amounts of inhibitor to each 20-μL aliquot of sample. We chose the minimum volume of inhibitor preparation that completely inhibited the amylase activity in the serum and in the standard, which in both cases corresponded to about 0.25 μL of inhibitor per unit of amylase per liter. We used twofold this volume (0.50 μL per U/L) to calculate the amount of inhibitor to be added. (This calculation should be repeated with each new lot to account for possible changes in inhibitor concentration.) Table 1 gives the instrument setting used in analyzing for total or remaining amylase activity with NPG 5/6 as substrate.

Calculation of amylase isoenzymes activity. Figure 1 shows the curve and the formulas used to calculate pancreatic (P) and salivary (S) amylase isoenzymes. The value for total (T) amylase was obtained in the absence of wheat-germ inhibitor. The residual (R) amylase activity, after adding inhibitor, was measured. We mixed human pancreatic (P) and salivary (S) standards in different ratios and measured its corresponding uninhibited fractions (R/T). We obtained the curve by plotting R/T against P/S ratios. The isoamylase activities in biological samples were taken from this standard curve or they were calculated by relating the degree of inhibition in the sample to that obtained in standards.

Results

By using our proportions of mixed standards and wheat-germ inhibitor we always obtained inhibition rates of 80 to 85% for S amylase and 15 to 20% for P amylase. The inhibition rate of amylase inhibitor was constant in solutions having total amylase activities between about 20 and 900 U/L or greater. With every lot of wheat-germ inhibitor we have used up to now (four), we found that 0.50 μL of inhibitor per U/L of amylase in the untreated sample sufficed for complete inhibition under our conditions.

The standard curve we obtained by plotting the logarithms of the P/S ratios (pure human pancreatic amylase/pure human salivary amylase in the range from 0.1 to 9.0) against the remaining fractional amylase activity (R/T) was linear for P/S ratios from 0.2 to 5.

Figure 2 shows the linearity of isoamylase activity after inhibition with amylase inhibitor.

Precision. Table 2 shows the within- and between-run precision for isoamylase determinations. We used three different individual sera and a pool of sera in 10 replicates each for the within-run assay. For the between-run assay we used the same four sera on 12 different days.

Analytical recovery. To a human serum, we added known

![Graph](image_url)
amounts of salivary and pancreatic isoamylases and assayed. Table 3 shows the results.

Correlation. Figure 3 shows the correlation \((r = 0.99)\) between isoamylase as measured by our manual method and the Phadebas method. In a previous report (II Interna-
tional Congress on Automation and New Technology in the Clinical Laboratory, Barcelona, Spain, 1984) we correlated the remaining fractional amylase activities obtained with our manual method and by our automated method. Again, the correlation coefficient was 0.99.

Reference interval. The reference values for serum iso-
amylase activity were obtained from data on 180 healthy
adults under 59 years old and 115 healthy persons over 60
years old. Total, S-, and P-amylose activities were 95 (SD
19), 33 (SD 14), and 29 (SD 10) U/L in the younger adults,
and 79 (SD 27), 45 (SD 23), and 35 (SD 13) U/L in the aged.
The values for total, S-, and P-amylose activity were signifi-
cantly different by Student’s t-test \((p < 0.001)\) between
younger adults and the aged.

Discussion

The wheat-germ inhibitor of \(\alpha\)-amylose has a much
greater specificity for human salivary than for pancreatic
isoamylase (4), which allows their differentiation.

We did not obtain, for every proportion of P- and S-
amylase standards we assayed, a linear response in remain-
ing fractional amylase activity. This result agrees with the
finding of Okabe et al. (7) but disagrees with that obtained
by Huang and Tietz (8) and Hoek (9). This fact implies that
the standard curve \((R/T vs P/S)\) is useful when the \(P/S\) ratio
is lower than 0.2 or greater than 5 (as in mumps infection
or acute pancreatitis, respectively). In the linear region, we
used the \(b\) and \(a\) coefficients illustrated in Figure 1 to
calculate P- and S-type amylases.

Both the substrate NPG 5/6 and the substrate NPG-7 are
specific; they have a defined chemical structure and they are
used in a defined concentration (6). Because there is no
product released in the amylase reaction that is detectable
photometrically, the rate of substrate reaction has to be
monitored by means of an indicator reaction involving \(\alpha-
glucosidase\), which hydrolyzes the amylase reaction prod-
ucts to glucose and free \(p\)-nitrophenol. \(\alpha\)-Glucosidase (EC
3.2.1.20), which acts on short maltose, acts on NPG-4, the
main product (about 60%) of the amylase reaction with the
NPG-7 substrate, only to a very small extent, resulting in
accumulation of this product. Because poor substrates are
generally inhibitors (6), the accumulation of NPG-4 results
in an increasing inhibition of the amylase reaction. The fact
that only 30 to 40% of the amylase-reaction products could
be used by the indicator reaction diminishes the sensitivi-
ty of the NPG-7 test.

NPG 5/6 substrate produces only a very small amount of
NPG-4, not more than 5% of the total product (6). All the
other reaction products of NPG 5/6 are easily split by \(\alpha-
glucosidase\), with the result that the test shows nearly an equimolar stoichiometry (1 mol of substrate yields 1 mol of
\(p\)-nitrophenol). This means that units resulting from the
action of \(\alpha\)-amylose on NPG 5/6 are fully compatible with
the amylase activity given in U/L. On the other hand, most

Table 1. Within- and Between-Run Precision for
Isoamylase Determinations (U/L) In Serum

<table>
<thead>
<tr>
<th>Serum</th>
<th>Within-run</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>CV, %</td>
</tr>
<tr>
<td>Serum I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>44 ± 2</td>
<td>4.5</td>
</tr>
<tr>
<td>S</td>
<td>51 ± 1</td>
<td>2.0</td>
</tr>
<tr>
<td>T</td>
<td>95 ± 1</td>
<td>1.1</td>
</tr>
<tr>
<td>Serum II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>26 ± 1</td>
<td>3.8</td>
</tr>
<tr>
<td>S</td>
<td>13 ± 1</td>
<td>7.7</td>
</tr>
<tr>
<td>T</td>
<td>38 ± 1</td>
<td>2.6</td>
</tr>
<tr>
<td>Serum III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>323 ± 16</td>
<td>5.0</td>
</tr>
<tr>
<td>S</td>
<td>69 ± 5</td>
<td>7.2</td>
</tr>
<tr>
<td>T</td>
<td>392 ± 13</td>
<td>3.3</td>
</tr>
<tr>
<td>Pooled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>31 ± 1</td>
<td>3.2</td>
</tr>
<tr>
<td>S</td>
<td>32 ± 1</td>
<td>3.1</td>
</tr>
<tr>
<td>T</td>
<td>63 ± 1</td>
<td>1.6</td>
</tr>
</tbody>
</table>

P, pancreatic amylase; S, salivary amylase; T, total amylase. n = 10 in the
within-run assay and 12 replicates each in the between-run assay.

Table 3. Analytical Recovery

<table>
<thead>
<tr>
<th>Activity, U/L</th>
<th>Original</th>
<th>Added</th>
<th>Measured</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>P</td>
<td>S</td>
<td>T</td>
<td>P</td>
</tr>
<tr>
<td>I</td>
<td>39</td>
<td>54</td>
<td>93</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>39</td>
<td>54</td>
<td>93</td>
<td>18</td>
</tr>
<tr>
<td>III</td>
<td>39</td>
<td>54</td>
<td>93</td>
<td>10</td>
</tr>
</tbody>
</table>

P, pancreatic amylase; S, salivary amylase; T, total amylase.
of the α-amylase methods involving oligosaccharides as substrate detect the salivary isoenzyme more sensitively than the pancreatic amylase. With NPG 5/6 substrate this effect is reduced but cannot be neglected. For all these reasons, we prefer the use of NPG 5/6 instead of NPG-7 substrate, which was recently reported (10).

This procedure can also be coupled to an automated kinetic system. We used an Hitachi 705 discrete analyzer system. This analyzer takes a sample volume no greater than 20 μL. If we used in this analyzer the same volume of inhibitor as in the manual method, the final dilution of the sample would be 60-fold that obtained in the manual method. Such a great dilution would shift the absorbances from the zone of greatest photometric accuracy and thus would decrease the test sensitivity. To obviate this problem, we adapted the inhibitor volume (reconstituting the wheat-germ inhibitor according to manufacturer’s instructions) to the total amylase activity in the sample (see Procedure).

Taking into account that the sample is prediluted with the adequate volume of inhibitor, the activity measured by the Hitachi 705 has to be modified according to this predilution. On the other hand, using less wheat-germ inhibitor reagent makes the isoamylase determinations less expensive.

Disease of the parotid glands is usually seen as inflammation, as in mumps infection. Sometimes the virus may involve the pancreas, an event that may be more frequent than previously thought (10). This can now be easily detected as an increase in both S-type and P-type amylase activity in the serum. Hyperamylasemia of the S-type may also be due to disease of the ovary or fallopian tube, or to a tumor (11). Investigations of such disorders may be greatly extended by the use of the inhibitor method. Diseases of the pancreas are reflected by changes in serum P-type amylase. Decreases in concentrations of P-type amylase occur in Swachman’s syndrome, cystic fibrosis, and in pancreatic insufficiency, increases during acute inflammation of the pancreas. P-type amylase has also been found in tumors. In an emergency, a single differential assay of amylase isoenzymes may be of most value as an aid to the diagnosis of acute pancreatitis.

References