Interferences with the Starch–Iodine Assay for Serum Amylase Activity, and Effects of Hyperlipemia

Peter H. Gliltz¹ and Christopher S. Frings²

Recent reports imply or claim that amylase activity is inhibited in hyperlipemic sera, because diluted samples showed greater activities when assayed by a starch–iodine method. We find that dilutions of both clear and lipemic samples give higher-than-expected activities when assayed by a starch–iodine method, an effect attributable to the variable effects of protein, turbidity, and triglycerides in the hyperlipemic samples. Thus the starch–iodine method is unsuitable for assessing the effects of hyperlipemic samples on amylase activity. To do so, we used an alternative method, in which soluble dyed amylpectin is used as the substrate. This method exhibits apparent zero-order kinetics, and we detected no interfering factors. Plots of sample volume (x) vs. activity (y) for clear and hyperlipemic (triglycerides up to 80 g/liter) sera gave straight lines with y-intercepts of zero. Evidently hypertriglyceridemia does not inhibit amylase activity.

Additional Keyphrases: pancreatic disease  •  amylolclastic procedure vs. chromogenic procedure

Abnormally high serum amylase activity is considered to be of diagnostic significance in cases of acute pancreatitis. The most popular methods of analysis are amylolclastic procedures involving the starch–iodine reaction (1, 2), a type of method that is usually suitable for clear samples, but becomes less satisfactory in the case of hyperlipemic samples because the increased turbidity can interfere with the final absorbance measurement. Also, as Farmer et al. (3) have noted, hyperlipemia may oftentimes accompany pancreatitis, which lends more importance to the accurate assay of amylase in hyperlipemic specimens. Thus reports of amylase activity being suppressed in lipemic sera (4–6) when assayed by the starch–iodine methods seemed to us to merit further attention.

We studied the effects of clear and hyperlipemic sera on results by the starch–iodine method of Caraway (2) and conclude that this method is not valid for use with diluted or hyperlipemic specimens.

We also found an amylase method (7) that is not subject to interference when hyperlipemic sera are assayed. In this method, dyed amylpectin is used as a substrate, and it is suitable for ascertaining whether or not amylase activity is suppressed in hyperlipemic samples. We conclude that it probably is not.

Materials and Methods

Starch–iodine amylase method. We used the method of Caraway (2), but with two sources of reagents. One set of reagents was prepared in our laboratory by following reagent preparation instructions in reference 2, except that potassium fluoride was not added to the working iodine solution. We obtained the second set of reagents from Harleco, Gibbstown, N.J. 08027. All absorbance measurements were made with a Model 300 N spectrometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio 44074) with a 1.0-cm cuvette.

Dyed-starch methods. Two methods were evaluated for usefulness for assaying lipemic serum samples. The first was “Amylochrome” (Roche Diagnostics, Division of Hoffmann-La Roche Inc., Nutley, N. J. 07110) (8). The second dyed-starch substrate method was “Dyamyl-L” (General Diagnostics, Division of Warner-Lambert Pharmaceutical Co., Morris Plains, N. J. 07950) (7, 9). For the reagent blank in the Dyamyl-L method we used a 70 g/liter solution of bovine serum albumin in NaCl (9.0 g/liter). The sample is added to a solution of soluble dyed amylpectin and the mixture incubated at 37.0 °C for 10 min. The reaction is stopped by addition of a precipitant (1 g of zinc chloride and 0.1 mol of benzoate per liter of 2-ethoxethanol/water, 82/18 by vol) that precipitates unhydrolyzed starch substrate and sample proteins. Absorbance of the supernate is proportional to amylase activity after subtracting the blank absorbance. The Dyamyl-L amylase test does require the presence of protein in the reaction mixture to help coprecipitate the unhydrolyzed substrate. All absorbance measurements were made with a Spectronic 70 spectrophotometer (Bausch & Lomb, Analytical Systems Division, Rochester, N. Y. 14625) with a 12-mm cuvette.

Interference studies. To test the interference of serum samples on the starch–iodine assay, we devised the following procedure. Five milliliters of starch reagent was added to a 50-ml graduated tube and incubated for 5 min at 37 °C. To this, 0.10 ml of serum or diluted serum was added, the contents mixed, and 5.0 ml of iodine reagent added immediately. The resulting solution was diluted to a volume of 50 ml with water and the absorbance measured at 660 nm against water. A starch–iodine blank was also run so that its absorbance could be used in subsequent calculations.

To test interference of serum samples on the Dyamyl-L test, we followed the prescribed procedure, except that no time was allowed for incubation. Instead, the precipitant was added immediately after the sample

¹ Medical Laboratory Associates, A Damon Laboratory, 1025 South 18th St., Birmingham, Ala. 35205.
² Postdoctoral Trainee in Clinical Chemistry.

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was added. A reagent blank was also run for this series of tests.

**Results**

**Effects of dilutions and interferences in the starch–iodine assay.** Figure 1 illustrates the effect of enzyme dilutions on activity in a clear sample of serum, with use of reagents from the two sources. The relationship between enzyme concentration and activity is linear; however, there appears to be significant "residual activity" when the lines are extrapolated to zero enzyme concentration. This leads to higher calculated activities as the samples are diluted. For instance, in the case for the Harleco reagents, 80 μl of sample gives a calculated 479 units of amylase activity, whereas 5 μl gives a calculated 1720 units. Apparently, serum samples contain factors that block the complexation of the starch and iodine so that lower absorbances result (factitiously higher activities). We have not identified all of the interfering factors, but protein is certainly a major one.

Table 1 shows kinetic data for the assay of a solution of human albumin (80 g/liter) for amylase activity. Whereas the reagent blank has an absorbance of 0.492, the albumin sample has an average absorbance of 0.430, with standard deviation of 0.003. This indicates that the interference from protein is instantaneous, and the result absorbance change is equivalent to 101 units of amylase activity on the basis of a 7.5-min time interval. This represents spurious activity, which in a real sample with comparable protein concentration would be reflected as higher activity. The line labeled "interference" in Figure 1 shows spurious activity partly caused by protein interference in dilutions of a clear serum sample. Units of activity were calculated on the basis of a 7.5-min time interval after testing for interference as described in *Materials and Methods*. Computations were based on the assumption that without "real amylase activity" the zero time absorbance change would remain constant as was found for the albumin solution (Table 1).

The effect of protein interference cannot be completely removed by dilution. Spurious activity with 100 μl of sample is 78 units, whereas, a 20-fold dilution (5 μl) shows 35 units and even a 100-fold dilution shows 28 units. Apparently, protein even in very low concentrations can block the interaction of iodine with starch, possibly through preferential binding of iodine by protein.

Figure 2 illustrates the effect of enzyme dilutions on activity in two extremely lipemic serum samples. The lines exhibit either linearity or nonlinearity, depending on the particular sample and source of reagents. In the sample showing a linear relationship between dilutions of the sample and units of activity, higher calculated activities result as greater dilutions are analyzed, with an apparent residual activity of 67 units. In the sample showing a nonlinear relationship, greater dilutions also result in higher calculated activities, but evidently there are additional interactions. The line designated as interference in Figure 2 measures the spurious activity from instantaneous interference in dilutions of one hyperlipemic sample (triglycerides, 70 g/liter). Units of activity were computed on the basis of a 7.5-min time interval.

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**Table 1. Time Course Study for the Assay of Albumin by the Caraway Method for Apparent Amylase Activity**

<table>
<thead>
<tr>
<th>Time, s</th>
<th>Absorbance (880 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent blank</td>
<td>0.492</td>
</tr>
<tr>
<td>10</td>
<td>0.432</td>
</tr>
<tr>
<td>60</td>
<td>0.430</td>
</tr>
<tr>
<td>120</td>
<td>0.430</td>
</tr>
<tr>
<td>240</td>
<td>0.433</td>
</tr>
<tr>
<td>360</td>
<td>0.434</td>
</tr>
<tr>
<td>480</td>
<td>0.426</td>
</tr>
<tr>
<td>600</td>
<td>0.426</td>
</tr>
</tbody>
</table>

*At zero time, albumin solution (80 g/liter) was added to the starch substrate. Iodine solution was added after the stated time intervals. The albumin we used was purified human albumin from Dade, Division of American Hospital Supply Corp., Miami, Fla. 33152.*

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Fig. 1. Effect of dilutions on amylase activity in a clear serum sample assayed by the starch–iodine method at 7.5 min and zero time (interference line)

Dilutions of the serum samples were made using NaCl (9.0 g/liter); 100 μl represents the undiluted sample. Harleco reagents. in-house reagents. △, interference line as described in text; average values on using the two sets of reagents (the data points were almost superimposable) and one lipemic specimen (triglycerides, 70 g/liter)

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Fig. 2. Effect of dilution on amylase activity in hyperlipemic samples assayed by the starch–iodine method at 7.5 min and zero time (interference line)

Serum samples were diluted with NaCl (9 g/liter); 100 μl represents the undiluted samples. ●, Triglycerides = 70 g/liter, in-house reagents. △, Triglycerides = 70 g/liter, Harleco reagents. ○, interference line as described in text; average values on using the two sets of reagents (the data points were almost superimposable) and one lipemic specimen (triglycerides, 70 g/liter)
interval after testing for interference as described in Materials and Methods. The slope of this line is opposite to that which is obtained for a clear sample (Figure 1). The divergence in the slopes between the clear and lipemic samples is accounted for in part by the turbidity of the hyperlipemic sample. Turbidity increases absorbance, so the apparent activity would be instantaneously decreased; but the turbidity effect does fall off linearly, as determined in our laboratory.

Dyed-starch amylase assays (chromogenic assay). We first used “Amylochrome” (8) to assay an extremely lipemic serum sample (triglycerides, 50 g/liter). The final solution after centrifugation remained very turbid with lipid-like material suspended at the meniscus. This preliminary experiment precluded further use of Amylochrome, which is no improvement over the starch-iodine method for assay of amylase in hyperlipemic samples.

Figure 3 shows the kinetics of the Dyamyl-L assay (7, 9) for a clear sample and an extremely hyperlipemic sample. The apparent zero-order kinetics of this enzymatic assay makes it suitable for confirming the presence of an inhibitor. The results of dilutions of a clear sample and two hyperlipemic samples are shown in Figure 4. These data indicate that there is no interference from hyperlipemia when the Dyamyl-L Amylase test is used and that amylase activity is not suppressed in extremely hyperlipemic sera. Moreover, even though original hyperlipemic serum samples were completely opaque, the final assay solutions after centrifugation appeared uniformly clear. The alcoholic precipitant effectively precipitates the serum lipoproteins and other serum proteins, as well as the unhydrolyzed dyed amylpectin.

Comparison of interfering factors in the starch-iodine method and the Dyamyl-L test. Table 2 presents the results of interference studies, performed as described in Materials and Methods, of three common interfering factors in serum specimens. The data corroborate the numerous interferences in the starch-iodine method. In icteric and hemolyzed serum samples, protein is probably the major interfering factor. However, in hyperlipemic samples, protein accounts for a smaller proportion of the interfering factors, and one must consider the additional effects of turbidity and triglycerides themselves. Extremes of these effects may be seen in two of the hyperlipemic specimens: the specimen with triglycerides of 19.5 g/liter demonstrates “inhibition” of the starch-iodine reaction by triglycerides whereas extreme turbidity of the specimen with a triglycerides concentration of 80 g/liter causes the apparent instantaneous activity to be negative.

As opposed to the starch-iodine method, the Dyamyl-L test is essentially free from interference in icteric, hemolyzed, and most importantly, lipemic sera. Additionally, because a protein solution serves as the blank, this automatically compensates for the presence of protein in the samples.

Discussion

Some reports have indicated that serum amylase activity may be “normal” in patients with hyperlipoproteinemia and pancreatitis. Specifically, Cameron et al. (6) found normal amylase activities in the hyperlipemic serum of patients with confirmed pancreatitis, and speculated that hyperlipemic serum may affect the laboratory procedure used for amylase determination or that an inhibitor of amylase may be present in the serum of these patients. Similar findings were reported by Havel (10) and Greenberger et al. (11). Fallat et al. (4) stated that high plasma triglyceride concentrations interfere in vitro with determination of amylase activity, and that serial dilutions of serum with saline allow correction to “actual” activity in hyperlipemic samples. Ramirez and Bates (5) use the method of dilutions in routine testing with the starch-iodine method for assay of amylase activity.

When the method of dilutions was repeated in our
Table 2. Interference Studies of the Starch–Iodine and Dyamyl-L Amylase Tests

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starch–iodine assay&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Starch–iodine assay&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dyamyl-L test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human albumin (80 g/liter)</td>
<td>102</td>
<td>93</td>
<td>—</td>
</tr>
<tr>
<td>Lipemic serum (triglycerides = 18.4 g/l)</td>
<td>18</td>
<td>39</td>
<td>-4</td>
</tr>
<tr>
<td>Lipemic serum (triglycerides = 19.4 g/l)</td>
<td>170</td>
<td>121</td>
<td>2</td>
</tr>
<tr>
<td>Lipemic serum (triglycerides = 11.2 g/l)</td>
<td>64</td>
<td>70</td>
<td>-4</td>
</tr>
<tr>
<td>Lipemic serum (triglycerides = 30.8 g/l)</td>
<td>94</td>
<td>-88</td>
<td>0</td>
</tr>
<tr>
<td>Icteric serum (bilirubin = 130 mg/l)</td>
<td>67</td>
<td>93</td>
<td>-1</td>
</tr>
<tr>
<td>Hemolyzed serum (hemoglobin = 1.0 g/l)</td>
<td>85</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Hemolyzed serum (hemoglobin = 5.0 g/l)</td>
<td>82</td>
<td>82</td>
<td>5</td>
</tr>
</tbody>
</table>

* Calculations were performed as if the assays were allowed their usual incubation times (7.5 min for the starch–iodine assay and 10 min for the Dyamyl-L test), even though the tests were terminated immediately. This approximates the false activity in an actual analysis.

* Reagents obtained from Harleco.

* Reagents prepared in our laboratory.

...is a very confusing situation arose. We did obtain higher values upon dilution of various hyperlipemic sera, but this phenomenon also pertained to clear samples. In attempting to clarify these findings, we confirmed the interference effects of protein and various other components (Figures 1 and 2, Tables 1 and 2) on the starch–iodine reaction. Interference by protein has been noted in several previous publications. In a study of the electrophoretic nature of human amylase, Wilding (12) showed that albumin is not associated with amylase activity, and that interference with the starch–iodine reaction accounted for the apparent reported activity. Searcy et al. (13) also investigated the interaction of human serum protein fractions with the starch–iodine complex and concluded that both albumin and γ-globulin dissipate the starch–iodine color.

As summarized above, the difficulties of assaying extremely hyperlipemic serum samples for amylase activity have recently been noted. Interferences with the starch–iodine method are manifold, including those from protein, lipoproteins (turbidity), and triglycerides themselves (Table 2). Proteins and triglycerides decrease the absorbance of the test solution, leading to spuriously higher values for activity, but the turbidity of the sample increases the absorbance, leading to falsely low activity. Depending on the particular sample, these components would have variable and unpredictable results. One serum sample (triglycerides, 30 g/liter) tested in our laboratory was so turbid that apparent activity after the usual 7.5-min assay was negative in the undiluted specimen. We conclude that assay of amylase activity by the starch–iodine assay is not accurate in any sample, and is completely unsuitable for assay of hyperlipemic samples.

Moreover, even in clear samples, the interference by protein with the starch–iodine reaction leads to a spurious background activity, which is variable but depends somewhat on protein concentration. This spurious activity has apparently been included in establishing normal values. Also, this background activity makes it invalid to dilute serum for assay of amylase activity by the starch–iodine method.

Alternatively, we have found that the Dyamyl-L test is suitable for assay of both clear and hyperlipemic sera, because the kinetics of the assay are linear and because it is free from the interferences discussed. Our data by this method suggest that hypertriglyceridemia has no effect on real amylase activity.

References