Automated Saccharogenic Assay of Alpha-Amylase Activity in Serum

Louis Fridhandler and J. Edward Berk

A saccharogenic amylase assay in which amylopectin is used as the substrate has been adapted for use with the AutoAnalyzer. Results closely matched those obtained with an established manual saccharogenic method.

Additional Keyphrases amylopectin as substrate • AutoAnalyzer • shellfish glycogen as substrate • macroamylasemia • dialysis step in analysis • gel chromatography • electrophoresis on paper • results by dinitrosalicylic acid method compared • hyperamylasemia

Continuous Studies in our laboratory on certain characteristics of amylase prompted us to seek a quantitatively reliable, automated saccharogenic assay method that would permit ready analysis of numerous samples. An automated iodometric amylase method has been described by Wilding (1). This method, however, is not quantitative and does not eliminate protein interference. The technique devised by Harms and Camfield (2) increases iodine concentration to substantially overcome protein interference, but depends on iodometric detection of starch disappearance. Recently, starch labeled with isotoic anhydride has been used as the basis for an automated fluorometric amylase method (3). Strumeyer and Romano (4) devised an automated saccharogenic assay that is highly sensitive but does not eliminate potential interference by protein with the estimation of reducing power.

The saccharogenic method we describe here has the advantage of substantially removing protein interference by means of dialysis, while still retaining adequate sensitivity.

Materials and Methods

This amylase assay is based on measurement of reducing power generated by hydrolysis of amylase-free amylopectin prepared from waxy maize (Calbiochem, Los Angeles, Calif. 90036).

Standardized hog pancreatic amylase solution was supplied by Calbiochem.

Reducing power of the samples, expressed as mg of glucose per 100 ml, was first assayed in the absence of substrate by pumping a “blank” or control solution through the system and allowing this to mix with the sample (30/h; sample-to-wash ratio, 2:1). A standard curve was constructed from the results obtained with glucose standards (2, 4, 8, 12, and 16 mg/100 ml) prepared in water saturated with benzoic acid.

The samples and standards were then run through the system again. This time, however, substrate solution rather than control solution was mixed with the samples. The reducing power found in the samples after incubation in the presence of amylopectin was likewise expressed as mg of glucose per 100 ml. The net increase in reducing power in the presence of substrate was then taken as the measure of amylase activity.

Preparation of Materials

Control solution. The following materials were added to a 2-liter volumetric flask:

(a) 160 ml of DMSO1 (Mallinckrodt Chemical Works, St. Louis, Mo. 63160).
(b) 800 ml of water.
(c) 400 ml of NaCl, 8.5 g/liter.
(d) 15.4 g of “Trizma-7.2” (Trizma preset-pH crystals; brand of Tris, Sigma Chemical Co., St. Louis, Mo. 63118) dissolved in 160 ml of water; final concentration of Tris was 50 mmol/liter, and pH at 40°C was 6.8. In some experiments, “Trizma-7.5” was used (15.06 g) to attain the same Tris concentration, but with a final pH of 7.1 at 40°C.
(e) Water to a final volume of 2 liters.

From the Department of Medicine, University of California at Irvine, Irvine, Calif. 92664.
Received July 6, 1970; accepted Aug. 31, 1970.
1 Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DMSO, dimethylsulfoxide; SU, Somogyi units; and DNSS, 3,5-dinitrosalicylic acid.
Finally, 2 ml of a 30 ml/100 ml solution of Brij-35 (Technicon Corp., Tarrytown, N.Y. 10591).

Substrate solution (500 ml). Place 4 g of amylopectin in a 250-ml beaker. Add 40 ml of DMSO, and immediately stir with a glass rod to prevent the amylopectin from caking. Heat the mixture gently, with continuous stirring, until the temperature reaches 80° to 83°C. The mixture becomes viscous and eventually clear, except for numerous entrapped bubbles. Add 50 ml of water (5 to 10 ml at a time) with constant vigorous stirring, followed by another 50 ml of water (25 ml at a time), and a final 50 ml of water. Transfer this well-mixed solution quantitatively to a 500-ml volumetric flask while adding a further 150 ml of water. To this solution, add 100 ml of NaCl, 8.5 g/liter. To provide buffering, dissolve 3.85 g of Trizma-7.2 (or 3.76 g of Trizma-7.5) in 40 ml of water, and add. Dilute with water to a final volume of 500 ml, and mix thoroughly. No wetting agent is added. Finally, heat this somewhat turbid substrate solution to 95°C for about 3 min, allow to cool, and store at 4°C. Reheat the solution once a week, and discard it after no longer than four weeks.

Procedure

The manifold is diagrammed in Figure 1. Sampler II and Proportioning Pump I (Technicon) were used. The 40°C heating bath had two glass coils, each 12.1-m in length and 1.6-mm i.d. The sample (mixed with substrate or control solution and air) was pumped through both coils in series, transit time being about 25 min.

Pore size of the dialyzer membrane was 4 to 6 nm. Once the sample-substrate stream entered the dialyzer, the reducing power in the stream was estimated as described in the standard AutoAnalyzer method for glucose (Method N-2b, Technicon Corp.), and the same potassium ferricyanide reagent was used here.

Proportioning Pump I was started. While other lines were aspirating water, the line identified as "Substrate or Control" (Figure 1) was placed in the control solution. To set the instrument on 100% transmittance, we pumped saline [NaCl, 8.5 g/liter, plus 0.5 ml of Brij-35 solution (300 g/liter) per liter] through the line marked "Ferricyanide." Ferricyanide reagent was then drawn through the same line.

Sampling was begun at least 30 min after the flow of control solution was begun, since the flow through the incubator and dialyzer did not become smooth and steady until the whole system was filled with control solution. Five minutes after the samples had all been aspirated for the first time, control solution was replaced by substrate solution. Not less than 10 min later, sampling was conducted for the second time. Each sample cup contained at least 2 ml of sample, which was sufficient for both samplings under these conditions.

The glucose standards served not only for constructing a standard curve, but also to provide marker signals, useful in confirming sample numbers of unknowns when the standards were placed strategically among the unknown samples on the sample tray. These standards were stable indefinitely since they contained benzoic acid. This preservative, however, could interfere with amy-
lase activity in the samples immediately following. To circumvent this, after each sample cup containing a standard we placed a cup containing saline buffered with 10 mmol of Tris, pH 7.0, per liter. This washed out any residual benzoic acid from the tubing. In some experiments we usedTris standards prepared in saline with 20 mmol Tris, pH 7.0, per liter, plus sodium azide, 0.2 g/liter. These did not interfere with amylase activity in samples immediately following. However, the long-term stability of such standards is not known.

**Exploratory Experiments**

Serum from a patient with pancreatitis, with an amylase activity of 1600 SU, was assayed with an established manual saccharogenic method with DNSA (8). It was then variously diluted with NaCl, 8.5 g/liter, and assayed with the AutoAnalyzer. This experiment demonstrated the relationship between amylase concentration and data yielded by our AutoAnalyzer assay system. Similar experiments were done with three other sera, containing 132, 332, and 1214 SU, respectively. These were similarly diluted with saline and then assayed with the AutoAnalyzer. Other diluted samples of these sera (1.4, 6, and 10 SU, respectively) were heated at 100°C for 15 min and then assayed with the AutoAnalyzer. A parallel experiment was carried out in which shellfish glycogen (Mann Research Laboratories, Orangeburg, N.Y.) was used as substrate instead of amylopectin.

Macroamylasemia is a recently recognized condition characterized by persistent hyperamylasemia and the presence in serum of a large molecular weight form of amylase (macroamylase) that cannot pass through the glomerular filter (8). Serum from such patients, when chromatographed through dextran gel, appears to contain not only the large molecular weight form of amylase but the normal size amylase as well. The elution pattern of these amylases was studied as follows:

Macroamylasemic serum was placed on a column (2.5 × 34 cm) of Sephadex G-200 (Pharmacia, Piscataway, N.J.). The column was eluted with Tris, pH 7.0 (20 mmol/liter in NaCl, 8.5 g/liter), and 5-ml fractions of the effluent were assayed for amylase, by both the manual DNSA method (8) and the AutoAnalyzer. Similar comparative studies were made with sera from two other patients with macroamylasemia.

Three samples of normal human serum were paper-strip electrophoresed in a Durrum cell (Spinco, Beckman) for 16 h at 90 V in Veronal buffer, pH 8.6, 0.071 mol/liter. A total of 0.45 ml of serum was applied to the origin of eight strips. The strips were combined and cut into 30 segments, which were then extracted with 5.0 ml of NaCl, 8.5 g/liter. Each extract was assayed by the manual and AutoAnalyzer techniques.

**Results**

A sample tracing obtained by AutoAnalyzer assay of (a) glucose standards (prepared with azide), (b) hog pancreatic amylase standards, and (c) human serum is shown in Figure 2.

Diluted sera heated to 100°C for 15 min showed no amylase activity in the AutoAnalyzer. When graded dilutions of serum with high amylase activity were subjected to assay with the AutoAnalyzer, there was a direct linear relationship between degree of dilution and reducing power (Figure 3).

![Fig. 2. Strip-chart record of AutoAnalyzer assay (with and without substrate)](image)

1-6, glucose standards (2, 4, 8, 12, and 16 mg/100 ml); 8 and 7, hog pancreas amylase (12 and 6 SU, respectively); 8 and 6, duplicate samples of human serum (180 SU) diluted 20-fold with saline

![Fig. 3. Relationship between results of the automated amylase assay and those obtained by a manual saccharogenic (DNSA) method with diluted samples of acute pancreatitis serum (1600 SU)](image)

Each point represents the mean of five determinations. The range about the means are indicated. Final pH in AutoAnalyzer incubation, 6.8
When this was repeated with three other sera whose respective amylase activities measured 132, 332, and 1214 su, the same linear relationship was noted in each case.

Substitution of glycogen for amylpectin (and use of serum with an amylase activity of 1600 su) indicated that shellfish glycogen is equally suitable as a substrate under these conditions. Indeed, reducing power was about 10% greater with glycogen.

The chromatographic elution pattern of macro-amylasemic serum shown by AutoAnalyzer assay was nearly identical to that revealed by the manual method (Figure 4). The peak positions were the same and the overall patterns were much the same except in the interval between the peaks.

The electrophoretic mobility of serum amylase was no different whether analysis was by the automated or the manual DNSA method (Figure 5).

Discussion

The automated saccharogenic amylase assay devised by Strumeyer and Romano (4) is limited in usefulness to highly purified amylase preparations since it does not include a dialysis step. While this omission increases sensitivity, it does not exclude ferri cyanide-reducing proteins, and this could increase the blank to unacceptably high levels. Dialysis was omitted by Strumeyer and Romano in the expectation that the hydrolysis of starch would initially yield oligosaccharides that would be too large to penetrate the membrane. Our apparently successful use of dialysis with amylpectin as the substrate indicates that products of enzymatic cleavage of amylpectin do pass through the dialysis membrane. Further, the amount of reducing products that penetrates the membrane is proportional to the enzyme activity within the limits shown.

The choice of amylpectin as substrate was influenced by several considerations. Use of amylase solutions poses a problem since these linear molecules may readily coalesce and precipitate, in contrast to amylpectin solutions (7). The use of amylpectin, therefore, allows larger amounts of

---

**Fig. 4. Representative data from one case, showing correspondence between chromatographic elution pattern shown by the automated method and that revealed by the manual DNSA method**

Final pH in AutoAnalyzer incubation, 7.1

---

**Fig. 5. Representative data from one case, showing correspondence between the electrophoretic mobility of amylase shown by the automated method and that revealed by the manual DNSA method**

Final pH in AutoAnalyzer incubation, 6.8
substrate solution to be stored with less risk of changes in dispersion that could alter susceptibility to enzymic hydrolysis, or result in precipitation. The interiors of linear amylase molecules are probably readily accessible to $\alpha$-amylase. Cleavage of unbranched amylase (200 to 1000 glucose units in length) therefore might well yield fragments too large to penetrate the membrane. Amylopectin, on the other hand, is extensively branched, the side chains being about 20 to 30 glucose units long. This configuration may at first shield its interior from attack by amylase. Thus, initial cleavage of the exposed, relatively short branches would be expected to yield hydrolytic products that are small enough to penetrate the dialysis membrane. In support of this, it has been reported that malto-triose and some other oligosaccharides are prominent among the initial products of the attack of salivary $\alpha$-amylase on amylopectin (8).

Amylopectin may be dissolved in water (at 82°C) without the use of DMSO. However, when DMSO is used to prepare the substrate solution, it is less turbid, which probably reflects a more complete dispersion of the amylopectin. Since this may enhance both susceptibility to enzyme and stability of the substrate solution, DMSO was regularly used in the preparation procedure. Previous studies established that this concentration of DMSO does not inhibit amylase activity (9).

A final pH of 6.8 to 7.1 would seem suitable in the AutoAnalyzer incubation step since values in this range are within the relatively broad pH optimum for amylase activity. However, the latter pH is not as close to the limit of the buffering range of Tris and, therefore, seems preferable for regular use.

Chromatographic and electrophoretic patterns are revealed by the automated assay with the same precision and reliability as with the manual method. The two methods differ, however, in several ways (Table 1). These differences deserve to be considered because, under other conditions, amylases from different tissues may react in significantly different ways with their substrate (8, 10). The question arises, therefore, whether variations in serum amylase shown under various conditions by the manual DNSA method would be paralleled by the data obtained with the AutoAnalyzer method. Although this question is un-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amylopectin、“Lintnerized” starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.05M Tris</td>
</tr>
<tr>
<td>Incubation temp.</td>
<td>40°C</td>
</tr>
<tr>
<td>Dialysis step</td>
<td>Yes</td>
</tr>
</tbody>
</table>

vestigated, it is of interest, especially since we observed that hyperamylasemia of two different types did not correlate when the results of two different assay methods were plotted against each other (11).

Supported by USPHS grant no. GM11897. Excellent technical assistance was provided by James R. Davis.

References