Macroamylasemia: Rapid Detection Method

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A reliable method is presented for rapidly screening human sera to detect macroamylasemia. A mixture of the serum to be tested, Blue Dextran, and cytochrome c is filtered through a small column of cross-linked dextran gel (Sephadex G-100). Amylase in the effluent fractions is detected by incubation with amylase and subsequent addition of iodine. Macroamylase, if present, is found in the blue-colored fractions, while amylase of normal molecular weight appears in the fractions colored by cytochrome c.

Additional Keyphrases gel filtration • cytochrome c and Dextran Blue as markers • diagnostic screening • hyperamylasemia

In 1970 we described observations made in our laboratory on 20 cases of “macroamylasemia,” and cited eight cases that had been reported to that time by others (1). Since then we have observed eight additional cases. That we should detect this number of cases in about five years suggests that this interesting but puzzling phenomenon is probably more prevalent than initially suspected.

Detection of macroamylasemia by the methods we and others have used is laborious and time-consuming (2), and also requires elaborate equipment that is not universally available. For these reasons, special studies of macroamylasemia have been confined essentially to patients with persistent, unexplained hyperamylasemia. It is entirely conceivable, however, that macroamylasemia may exist in persons who are either asymptomatic or have normal serum amylase activities. This possibility would be especially attractive and worthy of evaluation in relatives of patients with established macroamylasemia. Demonstration of macroamylasemia in such persons would alter present concepts regarding the relative frequency of this peculiar aberration and perhaps provide clues as to its origin and significance. It is desirable, therefore, to be able to detect macroamylasemia rapidly, easily, and reliably. This report describes a method that appears to meet these conditions.

Materials and Method

Reagents

Buffer. This is a mixture of 50 mmol of Tris [tris(hydroxymethyl)aminomethane], pH 7.2, 8.5 g of NaCl, and 0.2 g of sodium azide per liter.

Stock substrate solution (amylase, 50 g/liter). Amylase, 100 mg, (Nutritional Biochemicals Corp., Cleveland, Ohio 44128) is mixed with 2 ml of dimethylsulfoxide and dissolved with stirring at 80°C.

Working substrate solution (amylase, 1 g/liter). 0.5 ml of the stock substrate solution is mixed with 24.5 ml of the buffer described above.

Stock iodine solution. A solution of 4 g of KI (J.T. Baker Chem. Co., Phillipsburg, N. J. 08865) in 50 ml of water. To this is added 100 mg of iodine crystals. The mixture is stirred until the crystals dissolve, then diluted to 100 ml with water.

Working iodine solution. Stock iodine solution, 20 ml, is mixed with 10 ml of glacial acetic acid and 170 ml of water.

Amylase-marker solution. Forty milligrams of “Blue Dextran” (Pharmacia Fine Chemicals, Inc., Piscataway, N. J. 08854) is dissolved in 2 ml of the buffer described above, and 20 mg of cytochrome c (“Type VI”; Sigma Chemical Co., St. Louis, Mo. 63118) is then added. The solution is gently stirred until the cytochrome c dissolves.

Microcolumns are constructed from commercially available “Trilute” columns (Ames Co., Elkhart, Ind. 46514). These columns resemble the barrels of disposable plastic syringes, and have an inner diameter of 1.2 cm and a capacity of 6 ml. Two such barrels are joined in tandem to form a single column, which is then filled with cross-linked dextran gel (“Sephadex G-100,” Pharmacia) suspended in the buffer solution. A porous plastic disc (supplied with the Trilute column) at the outlet retains the gel in the barrels. Buffer solution is allowed to flow through the column until the gel beads have settled. The outlet is then plugged with a plastic stopper. A porous plastic disc is next positioned on top of the gel column, so a sample can be applied to the column without disturbing the gel. The gel column is then com-

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pressed slightly by pressing the top plastic disc with a stainless steel forceps (Figure 1, A, B, and C). A suitable height for the compressed gel column is 8.5 to 9.5 cm. The space in the barrel above the column serves as a reservoir for buffer, which can percolate through the gel by gravity.

Procedure

A mixture of 200 \( \mu l \) of serum and 20 \( \mu l \) of the solution of amylase markers is prepared. The microcolumn is inverted to empty the reservoir, then supported in the upright position, and the stopper removed from the outlet. When the residual buffer has migrated into the top porous disc, 200 \( \mu l \) of the mixture of serum and amylase markers, drawn into an Eppendorf pipet, is immediately applied to the top disc. After all the sample has entered the disc, three or four drops of buffer are added and allowed to enter the disc. The reservoir is then completely filled with buffer.

As the buffer mixture flows through the column, the Blue Dextran quickly moves ahead of the cytochrome c. When the blue color reaches the bottom of the column (in about 6 to 10 min), the effluent is carefully examined for appearance of blue drops. The first two or three faintly blue drops are discarded, and four fractions of nine drops each (approximately 60 \( \mu l \) per drop) of the subsequent blue-colored effluent are collected in 4-ml polystyrene sample cups (as used with the “AutoAnalyzer,” Technicon Corp., Tarrytown, N. Y. 10591). The fractions are placed in an aluminum-block incubator at 37°C and 200 \( \mu l \) of working substrate solution is added to each fraction, the cups being swirled gently to assure mixing. This mixture is incubated for 10 min. Amylase action is then stopped and residual amylase is simultaneously detected by adding 1.7 ml of the working iodine solution.

The color developing after the addition of iodine may range from deep blue through purple, brown, and yellow, depending on the extent of amylase digestion. The Blue Dextran color in combination with the iodine color imparts a slight greenish tint to the first, and possibly the second fraction, but this does not interfere with interpretation of the results.

Interpretation. Evidence for macroamylasemia consists of the presence of a peak of amylase activity in or near the void volume; amylase activity is evidenced by unequivocal decoloration to brown or yellow in fractions 1 and 2, with lesser decoloration in fractions 3 and 4. Should there be any uncertainty, the fractions may be incubated for 50 min rather than 10 min at 37°C.

Exploratory Experiments

Examinations were conducted on 24 sera from 22 different patients who had been shown by column chromatography [with Sephadex G-200 or, in some cases, polyacrylamide gel (“Bio-Gel P-300,” Bio-Rad Laboratories, Richmond, Calif. 94804)] and sucrose-density-gradient ultracentrifugation to have macroamylasemia. The amylase activity in these sera ranged from 312 to 1171 Somogyi units as measured by a manual method (3). With 12 of these sera, the technique was used as described; for six sera, effluent fractions were incubated at room temperature with substrate; the remaining six sera were incubated at 37°C for a time approximately inversely proportional to the amylase activity.

Seven sera from patients in whom macroamylasemia was not suspected were also examined by this method. In none of these was there a peak of amylase activity located near the void volume. Thus, it is apparent that the method of Tarrytown will detect macroamylasemia with high specificity.
lasemia was not suspected, and whose serum amylase activities ranged from 70 to 1760 Somogyi units, were also subjected to the same test. However, these fractions were incubated under various conditions, ranging from 10 min at room temperature to 50 min at 37°C. The essentially qualitative test procedure under study disclosed the existence of a macroamylase in one of these samples. This serum was further studied quantitatively (Figure 2) by applying 0.4 ml to a column of Sephadex G-100 (1.5 × 10.2 cm) and assessing amylase activity in the effluent fractions by an automated saccharogenic method (2). To rule out the possibility that an artificial macroamylase could form from the binding of amylase to Blue Dextran, no Blue Dextran or cytochrome c was mixed with the serum. Thirty-three fractions (0.75 ml each) were collected. Protein was measured by absorbance at 280 nm.

Test of Reliability of Method

The reliability of the standard technique described under "Procedure" was tested by comparing results for 10 macroamylasemic sera and 10 nonmacroamylasemic sera (six hyperamylasemic, four normoamylasemic). The amylase patterns had previously been demonstrated by the behavior of the serum on gel filtration through large columns (2.5 × 33 to 35 cm) of Sephadex G-200.

Precautions were taken to ensure that the sera were not directly identifiable by those carrying out the tests. Aliquots of 200 μl of each serum were prepared by one person (A). These samples were placed in 2-ml polystyrene sample cups (Technicon). Each cup was fitted with a polyethylene cap marked by a number different from the sample number originally assigned. A second person (B), uninvolved as to the identity of the prepared samples, randomly selected the capped vials and replaced each numberedcap with a lettered cap and recorded the changes. The samples were then frozen until tested. Just before the thawed sample was applied to the microcolumn, 20 μl of the solution of amylase markers was added and mixed. A third person (C) screened 14 of the samples; B examined one sample; and A screened five samples, with the results being interpreted by C.

The similarity of the elution pattern of cytochrome c to that of normal α-amylase [mol wt, about 50,000 (4); α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1] was verified by special tests conducted on serum from a patient with pancreatitis that was shown by Sephadex G-200 filtration to contain only amylase of normal molecular weight. A sample of the serum (0.35 ml) was mixed with 0.65 ml of buffer and 2 mg of cytochrome c. This mixture was passed through a column (2.5 × 35 cm) of Sephadex G-100. Forty 5-ml fractions were collected. Protein and amylase were measured quantitatively as indicated above. Cytochrome c was measured by absorbance at 415 nm.

Sephadex G-100 very adequately separates the two forms of amylase. However, Sephadex G-200 would be expected to enhance separation of the higher molecular weight serum proteins. The ability of the microcolumn to supply definitive quantitative data was therefore tested by using a microcolumn (1.2 × 11.4 cm) containing Sephadex G-200 superfine. The outlet of the microcolumn was fitted with a no. 15 square-cut syringe needle, 2.1 cm long, mounted in an automatic drop counter. The outer surface of the needle was thinly coated with silicone vacuum grease to ensure proper formation and release of the drops of effluent. Serum (0.2 ml) from a patient with macroamylasemia (Case 21) was applied to the column. Fractions of 0.35 ml each were collected and diluted with 2.0 ml buffer. Protein was measured by absorbance at 280 nm and amylase by an automated saccharogenic method (2).

Results

In all of the 24 samples of macroamylasemic serum, the macroamylase component appeared in the void volume, with hydrolysis of the amylase substrate being maximal in the first two effluent fractions and progressively less in the next two fractions (3 and 4). In the three cases in which it was tested, the amylase of normal molecular weight appeared in fractions 11 and 12, coinciding closely with the peak of cytochrome c color intensity.

In the exploratory experiments, a macroamylase was consistently detectable in all cases studied, regardless of the indicated variability in incubation time and temperature.

Amylose digestion in the seven serum samples not suspected of containing a macromolecular form of amylase was not apparent in effluent fractions 1 to 4 when the incubation time was 10 min at 37°C. However, when incubation was extended to 50 min, amylase activity became detectable in fraction 4 in two of these cases; enzymatic activity in both of these cases was well above normal (980 and 1760 Somogyi units, respectively). In one of these seven samples, amylase activity appeared unexpectedly in the void volume after a 50-min incubation. The existence in this case of an unsuspected macroamylase, in addition to the usual amylase, was confirmed by quantitative assay of a separate series of chromatographic fractions (Figure 2).

In testing the reliability of the method, macroamylasemic and nonmacroamylasemic sera were correctly identified in each of the 20 coded samples of serum.

The elution patterns of the usual amylase of normal molecular weight and cytochrome c after
filtration through a microcolumn of Sephadex G-100 appeared to be very similar and possibly identical; both peaks also emerged close to each other when a large G-100 column and quantitative methods were used. The ratio of elution volume to void volume was 2.3 for cytochrome c and 2.4 for amylase of the normal molecular weight. These values contrast with the ratio of 1.0 that would be expected for macroamylase under such conditions.

Serum protein and amylase fractions were even more sharply separated by microcolumn filtration when superfine G-200 was used instead of G-100 (Figures 2, 3). The separation obtained compared favorably to that obtained with much larger columns (8). The results shown in Figures 2 and 3 represent recoveries of about 100% and 85%, respectively.

Discussion

Serum may be reliably screened for macroamylasemia and the results known within 30 min if a microcolumn of dextran gel (G-100) is used. Blue Dextran, added to the sample, provides a marker for the void volume, thereby identifying the important fractions to be analyzed for the presence of a macroamylase. When the added cytochrome c (identified by its red color) has emerged from the column, this indicates that all the amylase has also emerged, and that the column is ready for immediate re-use. Thus, no automatic collection apparatus is required. Using two microcolumns, one person can easily screen four serum samples for macroamylase in an hour.

Detection of unhydrolyzed amylase by color interaction with iodine, as in the present technique, is essentially qualitative. Nevertheless, wide variations in amylase activity may be clearly discerned. This capability is of value in interpreting the results. Thus, a peak of amylase activity in or near the void volume (fractions 1 and 2) constitutes both necessary and sufficient evidence of the presence of a macroamylase. Confirmation that there is indeed a peak of amylase in the void volume is obtained by a progressive drop in amylase activity in the effluent fractions immediately following (fractions 3 and 4).

The detection by the microcolumn method of a previously unsuspected macroamylase in the serum of a patient with pancreatitis is of great interest. This confirmed finding provides additional proof of the reliability of the rapidly performed method as a screening procedure, and also lends support to the suspicion that macroamylasemia may be more prevalent than was thought. The actual prevalence of the disorder may now be estimated by using the microcolumn to screen sera from normal persons, patients with various disorders, and relatives of those shown to have macroamylasemia.

The use of a microcolumn (1.2 × 11.4 cm) of G-200 (superfine) for quantitative amylase studies offers several advantages over the large column (2.5 × 33 to 35 cm) that we and others have generally used hitherto. First, 0.2 ml of the sample to be tested is sufficient, as compared to 1 to 2 ml required for the larger column. Second, the time needed to collect the complete series of fractions is about 2.5 h as compared with about 15 h when the larger column is used. Third, the shorter time reduces the possibility of alteration in unstable material.

A microcolumn of G-200 (superfine) is superior to G-100 for obtaining quantitative data. However, G-100 is clearly preferable for the rapid method here described because of the greater speed of filtration. Further, the macroamylase accompanies the Blue Dextran in the void volume in G-100, while it is eluted after Blue Dextran in G-200.

The elution position of the normally encountered amylase (mol wt approximately 50,000) (4) is similar to and slightly greater than that of cytochrome c (mol wt 13,000). This supplies further evidence that amylase probably interacts with the dextran in the column, which retards its elution.

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References