Macroamylases: Differences in Activity against Various-Size Substrates

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Hyperamylasemia caused by macroamylases can lead to the overdiagnosis of acute pancreatitis. We examined whether interference from macroamylase is less in assays that use high-molecular-weight rather than oligosaccharide substrates. We hypothesized that high-molecular-weight substrates would be sterically excluded from macroamylasic complexes and thus would be hydrolyzed less efficiently. Eighteen macroamylasic samples were assayed by using red-dyed amylopectin or blue-dyed starch as polysaccharide substrates or by using maltoheptaose or maltotetraose as oligosaccharide substrates. The oligosaccharic substrates gave comparable results ($y = 0.81x + 83$); we observed consistently lower activities for amylopectin than for maltotetraose ($y = 0.32x + 38$). We observed no bias among methods when nonmacroamylasic specimens were analyzed. The mechanism of this difference was examined by adding anti-human pancreatic amylase antibodies to hyperamylasic serum samples from patients with macroamylasic and purified human pancreatic or salivary isoamylases. In each case, polyclonal and monoclonal antibodies lowered amylase activity more in assays with complex polysaccharides than in those with oligosaccharides. The use of high-molecular-weight substrates diminishes interference, and detection of suspected macroamylasic may be possible through comparison of activities determined from automated methods that use different substrates.

Additional Keyphrases: hyperamylasemia · macroamylasemia · source of variation

Macroamylase, as first described by Wilding et al. in 1984 (1), is an enzymatically active circulating complex of amylase bound noncovalently to immunoglobulins or to polysaccharides. Its presence, which can persist for years, often leads to an increase in amylase activity in serum, because decreased glomerular filtration of the macromolecular amylase complex delays circulatory clearance (2). Macroamylasemia is usually considered a benign condition (3) that affects 1–10% of all individuals, depending on the population studied (4). Its presence has no diagnostic value and only rarely has macroamylasemia been reported to reflect a pathophysiological process (5). Hyperamylasemia due to macroamylasemia can mislead the clinician, however, when serum amylase is ordered as a diagnostic test for acute pancreatitis in patients with abdominal pain and vomiting. Diagnosis and treatment in clinically urgent settings would be improved if the interference from macroamylases could be decreased and if simple automated methods for detecting macroamylasic were developed.

Definitive confirmation that hyperamylasemia is caused by macroamylasemia requires electrophoretic or gel-filtration studies that are not routinely available in most clinical laboratories. Precipitation with polyethylene glycol has been advocated as a screening test for macroamylasic complexes (6), but no screening test has been widely used in clinical laboratories, because each has significant drawbacks that impede its routine use. We previously reported that macroamylasic samples hydrolyze blue-dyed starch poorly after capture using noninhibiting monoclonal antibodies against human pancreatic amylase (7). These observations led us to hypothesize that macroamylasic complexes, by virtue of their size, could have lower activity against large substrates such as starch or amylopectin relative to the smaller oligosaccharides that have become popular substrates for use in automated amylase assays. Evidence to support this hypothesis from Adachi et al. (8) is that 8 of 30 immunoglobulin A–linked macroamylases show significantly lower enzyme activity with oligosaccharides than with complex polysaccharides. In this study, we compare the activity of macroamylasic complexes against various-size substrates, evaluate the basis for observed differences in specific activity, and suggest practical ways of applying our findings to automated screening for macroamylasemia in the clinical laboratory.

Methods

Sources and Processing of Specimens

All macroamylasic samples used in this study were kindly provided by John O'Brien, Mayo Clinic Laboratories (Rochester, MN). Unused portions of macroamylasic samples collected over a 3-month period were shipped frozen to us and kept at $-80^\circ$C until used for these studies. Each serum sample had been assayed for total amylase activity by using maltotetraose (Beckman, Brea, CA) as substrate and had been documented as macroamylase by gel-filtration chromatography using Sephacryl S-200.

Nonmacroamylasic samples with elevated or normal amylase activities were obtained from Barnes Hospital Clinical Chemistry Laboratory (St. Louis, MO) within 1 week of collection. Serum samples were stored at 4 ºC in the clinical laboratory and frozen by us upon receipt until assayed for amylase.

Antigen Purification and Antibody Production

Human pancreatic and salivary-type isoamylases were used for antibody-binding studies after they had been purified to homogeneity as previously described...
(9). Monoclonal antibodies were produced against purified human pancreatic amylase as previously reported (7), and polyclonal antibodies were produced by injecting rabbits subcutaneously with purified human pancreatic amylase (100 μg) mixed with complete Freund's adjuvant as a primary immunization followed by subsequent boosts at 2-week intervals with the same mass of purified amylase mixed with incomplete Freund's adjuvant. Monoclonal antibodies collected in mouse ascites and polyclonal antibodies in rabbit serum were purified by affinity or ion-exchange chromatography before use. All monoclonal and polyclonal antibodies used in these studies cross-react with human salivary isoamylases.

**Amylase Assays**

Serum samples were assayed for amylase activity by using four different substrates. Blue-dyed starch (Phadebas, Pharmacia, Piscataway, NJ) was used as previously described (9). Slides impregnated with Dri-marene Red Z2B amylopectin (Kodak Ektachem, Rochester, NY) were used according to the manufacturer's instructions on an Ektachem 700 analyzer. Glucose oligosaccharides in the form of maltotetraose (Electro-Nucleonics, Fairfield, NJ) or maltotetraose (Beckman) were used as substrates in the other two assays. For assays that used maltotetraose paranitrophenol (G₄), 25 μL of sample was mixed with 500 μL G₄ substrate and incubated for 10 min at 37 °C. The reaction was stopped with 200 μL Na carbonate 0.5 mol/L, pH 11, and absorbance was read at 405 nm with a Cobas-Bio Analyzer (Roche Diagnostics, Nutley, NJ). Maltotetraose (Beckman DS reagents) was used in an automated assay on a Technicon RA-1000 analyzer. Activity was assigned by running 0 and 700 U/L standards in parallel.

**Results**

**Comparison of amylase activity in macro- and non-macroamylaseemic samples using polysaccharide and oligosaccharide substrates.** Eighteen macroamylaseic samples were assayed by using either amylopectin (a complex polysaccharide) or maltotetraose (an oligosaccharide) as substrate, and the results were compared with those obtained by using maltotetraose (an oligosaccharide). Lower activities were obtained for each macroamylaseic sample with amylopectin than with maltotetraose (Figure 1). On average, amylase activity measured with the high-molecular-weight substrate was ~60% lower than the activity measured with the oligosaccharide substrates. Seventeen of the 18 macroamylaseic samples had activities that were >40% lower when the amylopectin substrate was used. In contrast, nonmacroamylaseic samples containing a broad range of amylase activities did not show the same substrate bias observed with macroamylaseic samples; similar catalytic activity was observed regardless of the size of the substrate used (Figure 1). Furthermore, the 18 macroamylaseic samples had similar catalytic activity toward the two oligosaccharide substrates maltotetraose and maltotetraose (y = 0.81x + 83).

**Effect of polyclonal antiamylase antibody on activity of hyperamylaseic sera.** To define more clearly the mechanism of this differential substrate activity observed with macroamylaseic complexes, the effect of polyclonal antibody produced against human pancreatic amylase (which cross-reacts with human salivary amylase) on amylolytic activity was investigated. Figure 2 shows the effect of polyclonal antibody on amylolytic activity in markedly hyperamylaseic (but nonmacroamylaseic) serum. With increasing antibody concentrations, progressive diminution in catalytic activity was observed regardless of the substrate involved. Much lower concentrations of antibody were required for the inhibitory effect, however, when either amylopectin or starch was used as substrate. Antibody concentrations of 50 mg/L completely inhibited enzyme activity in the presence of starch as substrate, but the same antibody concentration had little effect on activity when maltotetraose served as substrate. Similar inhibitory patterns were observed when nonmacroamylaseic serum was used, with only a mild elevation in total amylase activity (data not shown).

**Effect of polyclonal antiamylase antibody on activity of purified human pancreatic and salivary isoamylases.** Although the mean isoenzyme composition of macroamylaseic samples has recently been reported to be similar to that present in normal serum (10), earlier studies showed that, among individuals, the isoenzyme composition of macroamylaseic samples determined by fractionation varies widely (11). To determine whether the substrate dependence of the activity of antigen–antibody complexes is isoenzyme specific, human neonatal serum, which normally exhibits undetectable or very low amylolytic activity, was spiked either with purified salivary or pancreatic isoamylase. Figure 3 shows similar inhibitory patterns of amylase activity for salivary and pancreatic isoamylase–antibody complexes. For both isoenzymes, the pattern of inhibition
Fig. 2. Effect of substrate on inhibition of amylase by polyclonal antibody
For samples from nonmacroamylasemic patients either with marked hyper-
amylasemia (A) or with normoamylasemia (B), the effect of different substrates
(•, maltoheptaose; A, blue-dyed starch; ©, amylase) on amylase activity
is shown in the presence of various concentrations of polyclonal antibody
against human pancreatic amylase.

Fig. 3. Effect of various concentrations of polyclonal antibody against
human pancreatic amylase on the activity of purified pancreatic (□) and salivary (©)
isoamylases is shown for different substrates.

Fig. 4. Effect of various concentrations of a noninhibiting monoclonal
antibody against human pancreatic amylase on the activity of
purified pancreatic (□) and salivary (©) isoamylases is shown for
different substrates.

The degree of inhibition of amylase activity observed with maltoheptaose substrate was less than
that observed with amylpectin substrate at 50 mg/L antibody.

Effect of monoclonal antibody on activity of purified human pancreatic and salivary isoamylases. In contrast
to polyclonal antibodies, which recognize multiple epitopes, monoclonal antibodies bind to a single anti-
genic site. A monoclonal antibody directed against human amylase was selected for study. Neonatal serum
was spiked with either purified pancreatic or salivary isoamylase as described above but then incubated with
the monoclonal antibody and assayed with amylpectin or maltoheptaose as substrate. At an antibody concen-
tration of 100 mg/L, both pancreatic and salivary amylase activities were inhibited by 60–70% when amyl-
pectin was used as substrate (Figure 4). In contrast to the polyclonal antibodies, no concentration-dependent
effect was observed. Monoclonal antibody concentra-
tions that were more than fivefold higher failed to confer
an added inhibitory effect. Furthermore, when malto-
heptaose was used as substrate, no inhibitory effect was
observed even at the highest monoclonal antibody concentra-
tions studied. A similar substrate-dependent pattern
of activity was observed when a different antihu-
man pancreatic amylase monoclonal antibody was used
(data not shown).

Discussion
In this study hyperamylasemic samples containing
macroamylasemic complexes were found to have differing measured activities that depended on the size of the
substrate used for analysis. All 18 of the macroamyl-
asemic samples demonstrated higher activity toward
smaller oligosaccharide substrates than toward complex
polysaccharides such as amylpectin and starch. This
substrate bias was not observed for nonmacroamyl-
asemic hyperamylasemic serum samples, which sug-
gests that the lower catalytic activity of the complexes
toward larger substrates could reflect decreased access of the active site of amylase to substrate because of steric hindrance.

This hypothesis is supported by the in vitro studies. Formation of large antigen–antibody complexes created by adding specific anti-amylose polyclonal antibody to hyperamylasemic (nonmacroamylasemic) serum impaired the ability of amylase to hydrolyze complex polysaccharides in a concentration-dependent manner. Much higher concentrations of antibody were required to inhibit hydrolysis of oligosaccharides. Addition of polyclonal antibody to purified human isoamylases demonstrated substrate-dependent patterns of inhibition that were similar to those observed with serum containing mixtures of salivary and pancreatic isoenzymes. This indicates that substrate bias is independent of the isoenzyme composition of macroamylases and is significant because macroamylasemic complexes contain various proportions of pancreatic and salivary isoamylases (11).

Two murine monoclonal antibodies to amylase also produced substrate-dependent inhibition of amylase activity. There was ~60% inhibition of the hydrolysis of an amylopectin substrate. In contrast to the polyclonal antibodies, however, no inhibitory effect was observed with the monoclonal antibodies when the smaller substrate maltotetraose was used. Another significant difference from the effect of the polyclonal anti-amylose antibodies was that the monoclonal antibodies failed to demonstrate a progressive inhibitory effect on amylase activity with increasing antibody concentration. Attainment of maximal effect with low concentrations of monoclonal antibody and the lack of progressive inhibition against amylopectin is consistent with the presence of a single antibody-binding site.

The addition of a murine monoclonal antibody to amylase produces differences in substrate use that are strikingly similar to those observed for macroamylasemic samples. Thus, the addition of excess monoclonal antibody to hyperamylasemic serum likely represents the best in vitro model of macroamylasemia because most of the antibodies that form circulating macroamylasemic complexes are reportedly monoclonal and in excess with respect to antigen (4, 12–14). On the basis of current work and the observation that up to 96% of 269 macroamylasemic samples contain excess antibody and undetectable amounts of unbound amylase (4), the great majority of macroamylases should show much lower activity with polysaccharide substrates than with oligosaccharide substrates. The magnitude of any given antibody’s inhibition of polysaccharide hydrolysis can vary depending on the proximity of its cognate epitope to the enzyme’s active site. Our macroamylasemic samples showed a greater substrate bias than has been reported by others (8, 15). The reason for this difference is unclear but may be related to variations in the type of macroamylase studied or in the conditions of the assay [e.g., sample dilution, buffer, temperature (16)]. Many different types of macromolecules, such as immunoglobulins G and A, polysaccharides, or glycoproteins that are not immunoglobulins, bind to amylase in macroamylasemic complexes (2), and it is the structural variation among the macromolecules binding to amylase that may affect the degree of inhibition of amylase activity. Studies aimed at characterizing the differences among binding molecules may help to explain the variations reported here and elsewhere (8, 15) in the proportion of macroamylasemic samples that show substrate bias.

Automated measurement of total serum amylase may involve the use of large polysaccharides or small oligosaccharides coupled to a chromagen such as para-nitrophenol. Many larger clinical laboratories with different types of automated equipment may use either type of substrate and must choose the most useful method. Data presented here indicate that automated assays that use complex polysaccharides are more likely to give lower (and possibly normal) values for macroamylasemic samples than are those assays that incorporate oligosaccharides. Hyperamylasemia due to macroamylasemia has no diagnostic importance, and its detection can misdirect the clinician when acute pancreatitis is suspected. Thus, complex polysaccharides could be the preferred substrate in automated clinical assays, because lower activities will be reported for macroamylasemic samples. Those laboratories with the ability to perform automated amylase assays that use large and small substrates (but without the resources to perform column chromatography or electrophoresis) may strongly suspect macroamylasemia as the cause of hyperamylasemia when wide disparities in values obtained by the two methods are observed.

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References
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