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Effect of Substrate Size on Immunoinhibition of Amylase Activity

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Immunoinhibition assays are hypothesized to work by antibodies blocking substrate access to enzyme active sites. To test this hypothesis, the inhibition of amylase isoenzymes by monoclonal and polyclonal antisera was assessed using substrates of varying sizes: chromogenic sustrates 3, 5, or 7 glucose units in length, novel synthetic macromolecular substrates, and starch. The synthetic macromolecular substrates consisted of small oligosaccharide substrates linked to an inert polymer that conferred a large size to substrate molecules as determined by gel filtration chromatography. When substrate size increased, amylase activity could be inhibited equivalently by antibody concentrations that are 10-fold lower. Progressively less polyclonal serum was required to inhibit amylase activity as substrate length increased from 3 to 5 to 7 glucose units and as size was increased by linkage to a polymer. Different effects of substrate size were observed with two monoclonal antibodies. One monoclonal antibody blocked amylase activity independent of substrate size, while another monoclonal antibody had little inhibitory effect except using starch as substrate. We conclude that use of larger substrates can expand the repertoire of inhibitory epitopes on enzymes and convert a noninhibitory antibody into an inhibitory one. J. Clin. Lab. Anal. 15:64–70, 2001.

Key words: amylase; immunoassay; enzyme immunoassay; amylase isoenzymes; chromogenic substrates; macromolecular substrates

INTRODUCTION

Binding of antibodies directly to an enzyme, or to haptens linked to an enzyme, often inhibits the catalytic activity of the target enzyme. Usually, the mechanism for immunoinhibition of enzymes is considered to be a steric blockade of access of substrate to the enzyme's active site (1–4) although, in some cases, allosteric mechanisms may apply (5). The ability of antihapten antibodies to inhibit the enzymatic activity of enzyme-hapten conjugates has been applied to measure many drugs and hormones using methods such as the enzyme multiplied immunoassay technique (EMIT) (1,6). Direct enzyme immunoinhibition assays have been applied to specifically measure activities of the MB isoenzyme of creatine kinase (7) and the pancreatic isoenzyme of amylase (8–10).

In the present study, we examined the effect of substrate size on the ability of an antibody to inhibit enzyme activity. As a model system, we looked at the ability of various anti-amylase antibodies to inhibit amylase activity using different-sized substrates. Amylase cleaves α-1,4-glucosidic linkages in polysaccharides containing 3 or more α-1,4-linked D-glucose units. Approximately 200 methods for assaying amylase activity have been described (11). Most amylase assays used in clinical laboratories employ substrates that are small oligosaccharides containing 3 to 7 glucose units with a chromogenic group such as p-nitrophenol (pNP) added to the reducing end. Such assays use an exohydrolase such as α-glucosidase as an auxiliary enzyme to release chromophores from mono- or disaccharides produced by amylase. Recently, 2-chloro-p-nitrophenyl-maltotrioside (G3ClpNP) was introduced as a "direct" amylase substrate that releases a chromophore without auxillary enzymes (12). Measurement of amylase activity in serum assists in the diagnosis and treatment of pancreatitis (11–13) but is not specific for pancreatitis. Measuring the pancreatic isoenzyme improves specificity in the diagnosis of pancreatic disorders, and many methods have been applied to selectively measure the pancreatic isoenzyme (11,14,15). Immunoinhibition assays that use antibodies to block the activity of the salivary isoamylase have become the most commonly used method (8–10).

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MATERIALS AND METHODS

Reagents

Purified human pancreatic and salivary amylases were purchased from Scripps Laboratories (San Diego, CA) and Sigma (St. Louis, MO), respectively. Genzyme Diagnostics (Cambridge, MA) provided 2-chloro-p-nitrophenol-α-D-maltotrioside (G3ClpNP), p-Nitrophenyl-α-D-maltopentaoside (G5pNP) was from Calbiochem (La Jolla, CA), 4,6-O-ethylidene p-nitrophenyl-α-D-maltoheptaoside (EtG7pNP), and recombinant α-glucosidase from *S. cerevisiae* were from Boehringer Mannheim (Indianapolis, IN). Amylopectin azure, molecular weight standards, and rabbit albumin were from Sigma. Polyclonal rabbit antiamylase immunoglobulin fractions were purchased from Sigma (antiserum 1) and Calbiochem (San Diego, CA) (antiserum 2). Two mouse MABs specific to salivary amylase, MAB 88E8 and MAB 66C7 (8–10), were provided by Roche Molecular Biochemical (Penzberg, Germany).

Methoxypolyethylene glycol (mPEG)-coupled G3ClpNP and EtG7pNP substrates were prepared by reacting 50 mmol/L mPEG 5000 isocyanate (Shearwater Polymers, Huntsville AL) with an ≈2-fold molar excess of the G3ClpNP or EtG7pNP in dimethylformamide /10% diisopropylethylamine for 16 hours at room temperature. The manufacturer reported that the polymer had an average molecular weight of 4,900 Da with a narrow size distribution (SD ± 100 Da) of the primary component and about 5% of the polymer occurred as dimer. mPEG-coupled substrates were purified by gel filtration on Sephadex G-50 in 0.1% acetic acid. Conjugation reactions attached mPEG to about 30% of EtG7pNP and 10% of G3ClpNP molecules.

Gel-filtration Analysis of mPEG-Coupled Substrates

To analyze the size of substrates by gel filtration chromatography, a 1.5 cm × 25 cm column of Bio-Gel P-60 (Bio-Rad Laboratories, Hercules, CA) in 50 mmol/L sodium phosphate, pH 7.0 with 10% acetonitrile was used. Fractions of 0.8 ml were collected and A_{305} was measured. Molecular weight standards were bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), cytochrome c (12,400 Da), and aprotonin (6500 Da).

Maximal Cleavage of Uncoupled and mPEG-Coupled Substrates

Substrates were diluted into assay buffer and absorbances were recorded between 250 and 500 nm. To determine the maximum amount of substrate that could be cleaved with amylase, substrates were incubated with 60 U/mL of salivary amylase. When EtG7pNP and mPEG-EtG7pNP were used, 4 U/mL of α-glucosidase were added. Assays were continued until there was no absorbance change over more than an hour.

Amylase Assays

Amylase assays were run on a Cobas Fara analyzer (Roche, Basel, Switzerland) at 37°C, and absorbance changes were monitored at 405 nm for 20 min. Assay buffer was 52.5 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.15, 87 mmol/L sodium chloride, 12.6 mmol/L magnesium chloride, 0.075 mmol/L calcium chloride. Specimens containing amylase were diluted in buffer containing 1 mg/mL rabbit albumin. Final reaction volumes were 200 μL. For immunoinhibition studies, antibodies were preincubated with amylase for 30 min at room temperature prior to the addition of substrate. Activity of stock solutions of amylase was measured with a Hitachi 917 analyzer Roche (Indianapolis, IN) which uses EtG7pNP as the substrate. Amylase assays using amylopectin azure were based on that of Rinderknecht et al. (16).

RESULTS

Analysis of Synthetic Macromolecular Substrates

In order to generate synthetic substrates of a larger size, new macromolecular substrates (macrosubstrates) for amylase were prepared by linking small chromogenic substrates—G3ClpNP or EtG7pNP—to one end of mPEG molecules of 4,900 Da. Gel-filtration analysis showed that both macrosubstrates had a relatively large size in solution that was comparable to globular proteins of about 30, 000 Da. Figure 1 shows the elution profiles of mPEG-G3ClpNP (Fig. 1A, squares), G3ClpNP (Fig. 1A, circles), mPEG-EtG7pNP (Fig. 1B, squares), and EtG7pNP (Fig. 1B, circles) from a column of Bio-Gel P-60. Both of the macrosubstrates eluted as a single major component slightly before the elution position of carbonic anhydrase (arrow labeled 2) which has a molecular weight of 29,000 Da. Each macrosubstrate had a minor component eluting before the main product. The minor component likely represents a small proportion of macrosubstrate with two mPEGs attached. No free substrates were detected in the macrosubstrate preparations. Uncoupled G3ClpNP and EtG7pNP eluted at slightly more than the total column volume, indicating that they adsorbed weakly on the column and making it impossible to estimate the size of the free substrates by this technique.

To determine the maximal amount of macrosubstrate and uncoupled substrate that could be cleaved by amylase, substrates were incubated with excess salivary amylase until no further increase in absorbance was observed for more than 1 h. The amount of free chromophore released was determined at 400 nm. Up to 90% of free G3ClpNP and EtG7pNP were cleaved while 20% of mPEG-G3ClpNP and 50% of mPEG-EtG7pNP were cleaved (data not shown). These results showed that the macrosubstrates served as amylase substrates, but only a subset of the macrosubstrate molecules were susceptible to cleavage. The coupling reaction to link mPEG to small substrates targets hydroxyl groups and some points of attachment of mPEG on the oligosaccharide will block the

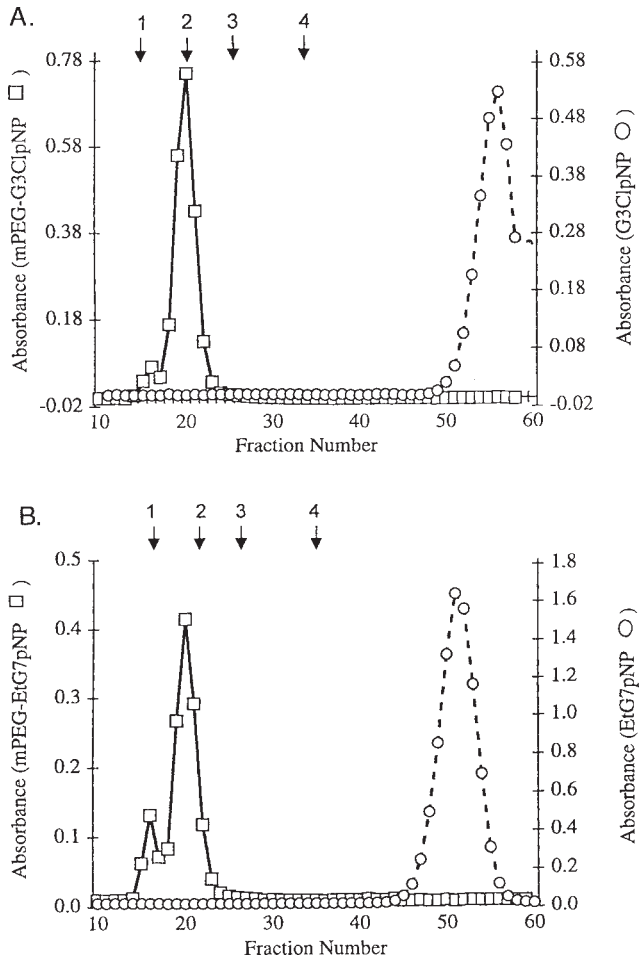

Fig. 1. Size-exclusion chromatography of amylase substrates. MPEG-G3ClpNP (**A**, squares), G3ClpNP (A, circles), mPEG-EtG7pNP (**B**, squares), EtG7pNP (B, circles) were analyzed on a column of Bio-Gel P-60 as described in MATERIALS AND METHODS. Elution of calibration standards is indicted by arrows: 1, bovine serum albumin (66 kDa); 2, carbonic anhydrase (29 kDa); 3, cytochrome C (12.4 kDa); 4 aprotinin (6.5 kDa).

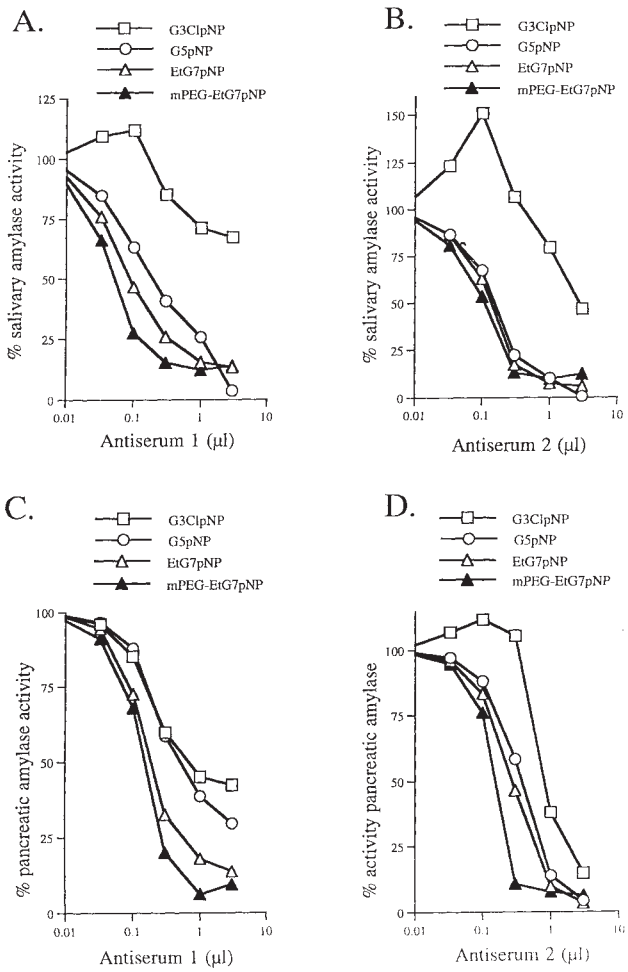

Fig. 2. Inhibition of salivary and pancreatic amylase activity by polyclonal antisera using varying size substrates. The indicated volumes of antiserum were preincubated with salivary (**A** and **B**) or pancreatic (**C** and **D**) amylase for 30 minutes before assay of activity with 0.8 mmol/L G3ClpNP, 0.4 mmol/L G5pNP, 0.4 mmol/L EtG7pNP, or 0.4 mmol/L mPEG-EtG7pNP. Final concentrations of salivary and pancreatic amylase were 20 and 40 U/L, respectively. One hundred percent amylase activity was determined in the absence of any antibody.

action of amylase or subsequent cleavage by α-glucosidase. Affinities of the synthetic macromolecular substrates were somewhat lower than homologous free oligosaccharide substrates. The apparent K_ms for the synthetic macrosubstrates (which are mixtures of different molecular forms) were 0.89 mmol/L for mPEG-EtG7pNP versus 0.13 mmol/L for EtG7pNP for salivary amylase, and 2.5 mmol/L for mPEG-G3ClpNP versus 2.0 mmol/L for G3ClpNP for salivary amylase at pH 6.5.

Effect of Substrate Size on Inhibition of Amylase Isoenzymes by Polyclonal Antisera

The effects of two different polyclonal antiamylase immunoglobulin preparations on salivary (Fig. 2A, B) and pancreatic (Fig. 2C, D) amylase activity using oligosaccharide substrates and mPEG-EtG7pNP were examined. Both antisera cross-reacted with salivary and pancreatic amylases due to high-sequence homology of the two isoenzymes (17), and the antisera inhibit amylase activity of both isoenzymes. The inhibitory potency of two antisera increased progressively with substrate size: mPEG-EtG7pNP > EtG7pNP > G5pNP > G3ClpNP. In most cases, the largest upperward step in potency was between the smallest substrate G3ClpNP and the next largest substrate G5pNP. Assays with EtG7pNP required about 50% more antibody to achieve the same level of inhibition as with mPEG-EtG7pNP. The overall change in potency from largest to smallest substrate ranged from a minimum of about 3-fold for antiserum 1 as an inhibitor of pancreatic amylase (Fig. 2C) to about 50-fold for either antiserum as inhibitors of salivary amylase (Fig. 2A,B). In most cases, it appeared that the antisera could approach 100% inhibition of

the isoamylases if enough antibody were added. However, in two cases, it appears that the inhibition of activity measured with G3ClpNP plateaued to yield a maximal inhibition of about 60% (Fig. 2A) or 40% (Fig. 2C).

Immunoinhibition studies using mPEG-G3ClpNP as a substrate were performed at 30- and 10-fold higher concentrations of salivary and pancreatic amylase, respectively, due to lower rates of cleavage of this substrate. Direct comparison of immunoinhibition of the mPEG-G3ClpNP and G3ClpNP showed about a 10-fold greater potency of inhibition of salivary amylase (Fig. 3A,B) and a 2- to 3-fold higher potency of inhibiting pancreatic amylase (Fig. 3C,D) using the macrosubstrate versus the small substrate. A plateauing of the inhibition of G3ClpNP was again apparent for inhibition of pancreatic amylase by antiserum 1. At the higher amylase concentration in these experiments, immunoinhibition of salivary amylase with the G3ClpNP did not reach a high enough level to observe whether inhibition plateaued.

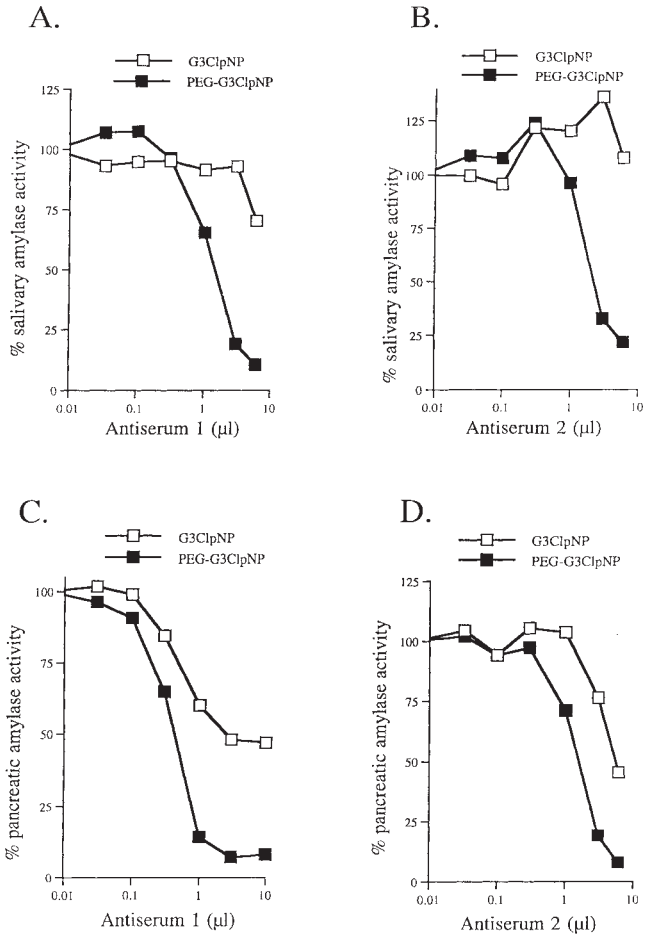

Fig. 3. Inhibition of salivary and pancreatic amylase activity by polyclonal antisera using G3-ClpNP and mPEG-G3ClpNP as substrates. Experiments were as in Figure 2 except for a change in substrate and use of higher enzyme concentrations–600 U/L pancreatic amylase and 400 U/L salivary amylase.

Effect of MABs on Pancreatic and Salivary Amylase Activity

Table 1 shows the percent of residual pancreatic and salivary amylase activity measured with different size substrates when excess MABs 88E8 and 66C7 were added. MAB 88E8 inhibited 93–98% of salivary amylase with all substrates and did not significantly alter pancreatic amylase activity. MAB 66C7 did not substantially alter salivary or pancreatic amylase activity with any of the substrates. A synergistic effect was seen with all substrates using a mixture of MABs 88E8 and 66C7, and together these antibodies inhibited 95–99% of salivary amylase activity. No significant effect on pancreatic amylase activity was seen using a mixture of MABs 88E8 and 66C7. There was little difference in the magnitude of enzyme immunoinhibition when different substrates were used.

Figure 4 shows the effect of increasing concentrations of MAB 88E8 on salivary amylase activity using EtG7pNP, PEG-EtG7pNP, and G3ClpNP as substrates. Salivary amylase activity was inhibited in a dose-dependent manner by this antibody, and no differences in antibody potency were seen between the three substrates tested.

Effect of MAB 66C7 on Amylase Activity Using a Starch Substrate

Figure 5 shows the effect of increasing concentrations of MAB 66C7 on salivary and pancreatic amylase activity using amylopectin azure as a substrate. Salivary amylase activity was inhibited in a dose-dependent manner by this antibody and salivary amylase activity was inhibited about 50% using 300 mg/L of MAB 66C7. Pancreatic amylase activity was unaffected by this antibody.

DISCUSSION

Previous studies showed that naturally occurring complexes of amylase with autoantibodies to the enzyme (macroamylase)

TABLE 1. Effect of MABs on salivary and pancreatic amylase activity using varying size substrates

Substrate	Amylase isoenzyme	Percent activity with MABs 66C7	88E8	66C7 + 88E8
G3ClpNP	Salivary	104	3	1
	Pancreatic	117	103	103
PEG-G3ClpNP	Salivary	128	2	0.3
	Pancreatic	104	101	101
G5pNP	Salivary	105	6	2
	Pancreatic	103	101	95
EtG7pNP	Salivary	105	6	2
	Pancreatic	105	96	96
PEG-EtG7pNP	Salivary	100	7	5
	Pancreatic	102	96	93

[a]Assays were performed as described in Materials and Methods. Final amylase concentrations were 20 U/L salivary and 40 U/L pancreatic for G5 and G7 substrates and 600 U/L salivary and 400 U/L pancreatic for G3 substrates. One hundred percent activity was determined in the absence of MAB.

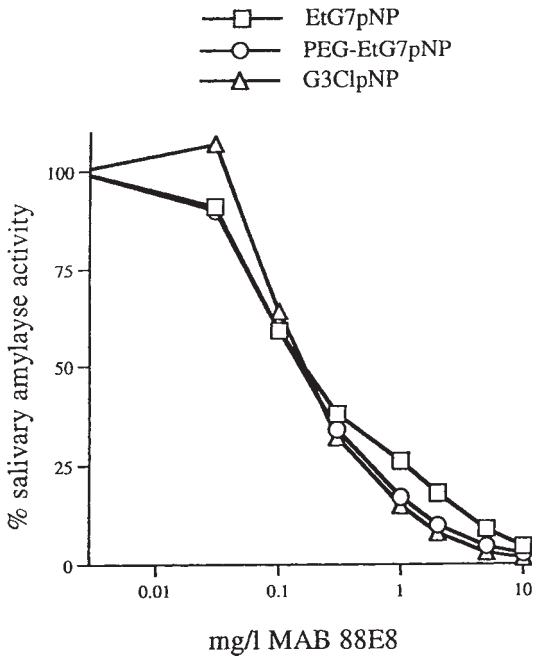

Fig. 4. Inhibition of salivary amylase activity by MAB 88E8 using EtG7pNP, mPEG-EtG7pNP, and G3ClpNP as substrates. Experiments were as in Figure 2.

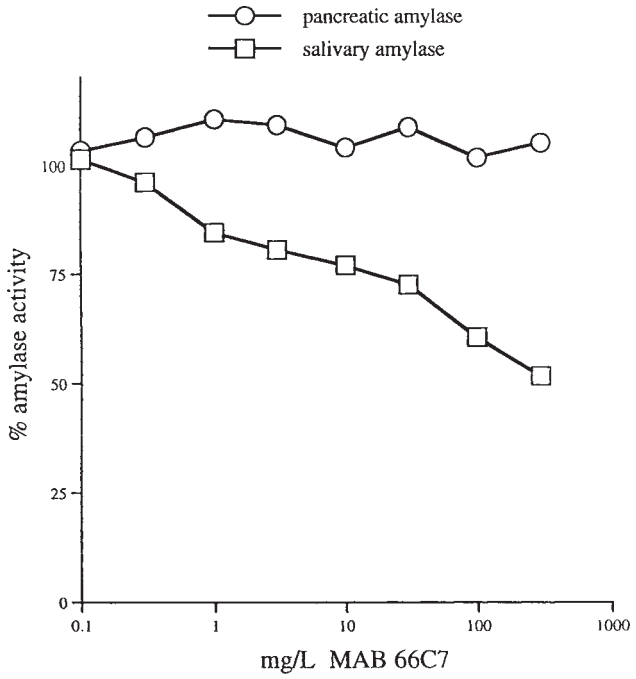

Fig. 5. Effect of MAB 66C7 on amylase activity using amylopectin azure as a substrate. Following preincubation of varying concentrations of MAB 66C7 with 200 U/L salivary (□) or 80 U/L pancreatic (○) amylase for 30 min at room temperature, amylase activity was determined using amylopectin azure.

have a lower level of activity when large polysaccharides such as starch and amylopectin are used as substrates than when small oligosaccharides are used as substrates (18). Antibody binding to amylase was proposed to exclude large substrates from access to the active site of amylase more efficiently than it does small substrates. Experience with the development of immunoinhibition assays using hapten-linked enzymes also suggested that these assays act by steric hindrance of antibody binding to the active site and that use of larger substrates can enhance immunoinhibition (1–4). Results of the present study provided further evidence that inhibition of enzyme activity by antibody binding can be enhanced by use of larger substrates. Synthesis of macrosubstrates, conjugates of small substrates with a polymeric carrier, served as a new tool for analysis of the effects of substrate size. Use of mPEG of 4,900 Da as the polymeric component formed macrosubstrates that had size-exclusion chromatography behavior similar to that of globular proteins of 30,000 Da, which have a radius of about 24 Å (19). The macrosubstrates thus behave as molecules only slightly smaller than amylase, which is a protein of 55,000 to 60,000 Da (17). mPEG has a large radius in solution relative to molecular weight because it has an extended random coil rather than a globular structure (20,21). The macrosubstrates are substantially larger than oligosaccharide substrates of amylase which are calculated to have effective radii of about 9 Å for G3ClpNP, 11 Å for G5pNP, and 12 Å for EtG7pNP, based on the formula of Squire (21). Even within the relatively narrow size range of the oligosaccharide substrates from 3 to 7 glucose units in length, there was a substantial size effect on inhibition of amylase by polyclonal serum. This effect occurred despite substantially higher affinity of the longer oligosaccharide substrates (22,23) that may lead to underestimation of substrate-size effects on immunoinhibition. Comparison of macrosubstrates with homologous small substrates may provide a more ideal evaluation of substrate size. The substrate components are identical, and the mPEG component of the macrosubstrate is a neutral space-filling group expected to have minimal interactions with enzyme or antibody (20).

The higher potencies of polyclonal antisera in inhibiting amylase activity when larger substrates are used is interpreted as an expansion of the number of epitopes that yield inhibition upon antibody binding. The results are consistent with the model diagrammed in Figure 6. Within a polyclonal antiserum, anti-enzyme antibodies fall into three classes: (1) antibodies binding to epitopes within the active site and inhibiting cleavage of all substrates; (2) antibodies binding to epitopes near the active site and inhibiting cleavage of large substrates but not small substrates. The size of this zone will depend on substrate size; and (3) antibodies binding to epitopes distant from the active site and exerting little steric effect on substrate access. Our experiments would suggest that use of a substrate with an effective radius of 24 Å rather than a substrate with a 9-Å radius leads to a substantial increase—up to 10-fold—in the number

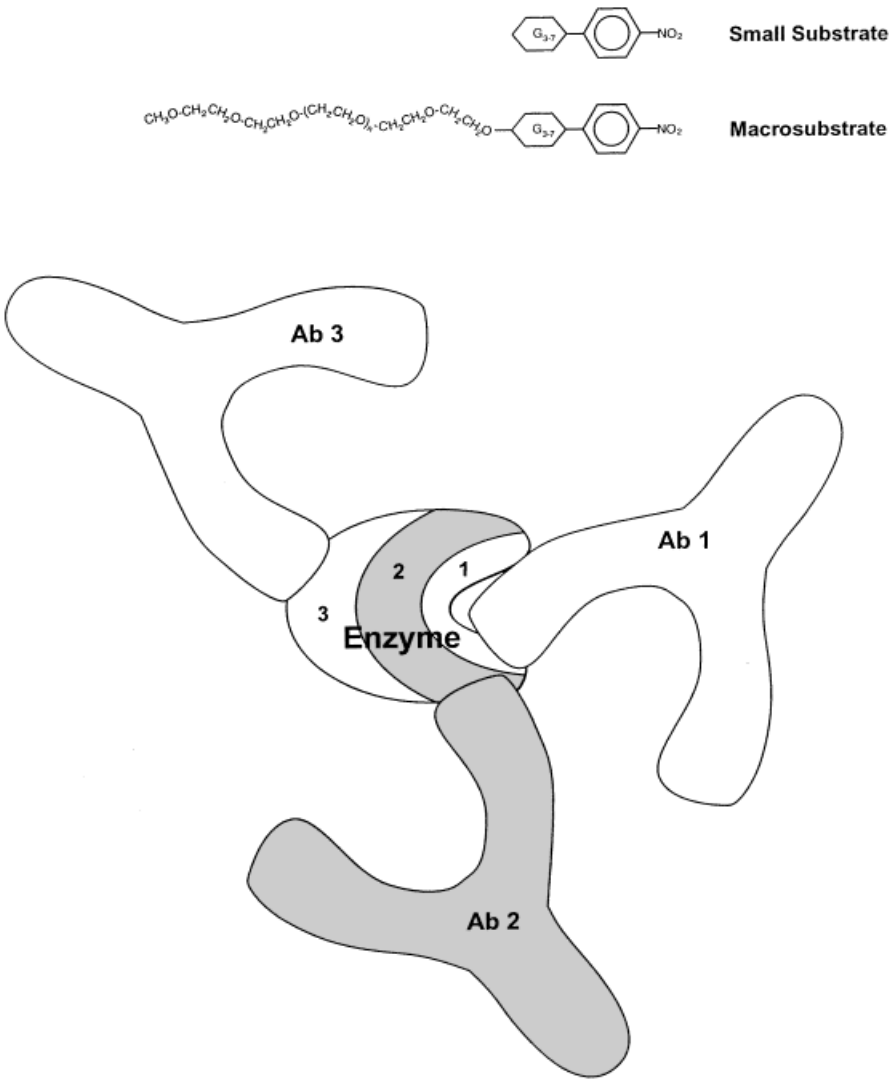

Fig. 6. Model of enzyme immunoinhibition with varying size substrates. Ab 1 represents antibodies binding to the active site of an enzyme (epitopes in zone 1 on the enzyme). All substrates are inhibited from reaching the active site. Ab 2 represents antibodies binding to epitopes neighboring the active site of the enzyme which sterically hinder macrosubstrates, but not small substrates from reaching the active site. Finally, Ab 3 represents antibodies that bind very far away from the active site and that do not sterically hinder access even of macrosubstrates.

of antibodies within a polyclonal antiserum that are inhibitory. A second factor that comes into play is that binding of some antibodies provides incomplete inhibition. With the G3ClpNP substrate, inhibition plateaued at about 40–60% in the presence of excess antibody. Use of a larger substrate provided more complete inhibition of activity.

Substrate size exhibited a different pattern of effect on the inhibition by two MABs that are specific for salivary amylase. No difference in antibody potency was noted for the inhibitory effect of MAB 88E8 regardless of the size of substrate used. MAB 88E8 most likely binds directly to the active site of salivary amylase (zone 1 of epitopes in Fig. 6) so that variation in substrate size does not alter its inhibitory effect. Without evidence of a substrate-size dependence, however, it is not possible to completely rule out an allosteric inhibitory effect.

MAB 66C7 serves as an example of the second class of antibodies that binds farther away from the active site of the enzyme. MAB 66C7 did not significantly inhibit salivary (or pancreatic) amylase activity using either mPEG-coupled or free oligosaccharide substrates. However, when very a large amylopectin substrate was used, MAB 66C7 specifically inhibited salivary amylase activity. MAB 66C7 most likely binds relatively far away from the active site of salivary amylase so that only very large substrates are sterically hindered from binding to the active site.

Results of the present study have general implications for the design of immunoinhibition assays. Use of the largest pos-

sible substrate usually is desirable in order to achieve the most complete inhibitory effect and to allow use of a broader spectrum of antibodies. Linkage of small substrates to an inert polymeric carrier to prepare macrosubstrates is identified as a means of preparing larger substrates. Use of a macrosubstrate rather than a small substrate may expand the range of inhibitory epitopes 10-fold or more. This may prove beneficial in identifying antibodies, aptamers, or other molecules to serve as enzyme inhibitors for diagnostic or therapeutic applications.

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REFERENCES

1. Rubenstein KE, Schneider RS, Ullman EF. "Homogeneous" enzyme immunoassay: new immunochemical technique. Biochem Biophys Res Commun 1972;47:846–851.
2. Cinader B. Antibodies to biologically active molecules. In: Proceedings of the second meeting of the Foundation of European Biochemical Societies. Vienna: Oxford Pergamon; 1965. 1:85–90.
3. Gibbons I, Skold C, Rowley GL, Ullman EF. Homogenous enzyme immunoassay for proteins employing β-galactosidase. Anal Biochem 1980; 102:167–170.
4. Skold C, Gibbons I, Russel ME, Juaristi E, Rowley GL, Ullman EF. Action of β-galactosidase on novel synthetic macromolecular substrates. A processive enzymic reaction controlled by coulombic interactions. Biochim Biophys Acta 1985;830:64–70.
5. Rowley GL, Rubenstein KE, Huisjen J, Ullman EF. Mechanism by which antibodies inhibit hapten-malate dehydrogenase conjugates. J Biol Chem 1975;250:3759–3766.
6. Rosenthal AF, Vargas MG, Klass CS. Evaluation of enzyme-multiplied immunoassay technique (EMIT) for determination of serum digoxin. Clin Chem 1976;22:1899–1902.
7. Ooi DS, Maddock MJ, Livesey JF, Donnelly JG. Creatine kinase-MB immunoinhibition in the diagnosis of suspected acute myocardial infarction. Clin Biochem 1996;29:497–500.
8. Gerber M, Naujoks K, Lenz H, et al. A monoclonal antibody that specifically inhibits human salivary α-amylase. Clin Chem 1987;33:1158–1162.
9. Rauscher E, Gerber M. Pancreatic χ-amylase assay employing the synergism of two monoclonal antibodies. Clin Chim Acta 1989;183:41–44.
10. Gerber M, Naujoks K, Lenz H, et al. Specific immunoassay of α-amylase isoenzyme in human serum. Clin Chem 1985;31:1331–1334.
11. Moss DW, Henderson AR. Enzymes. In: Burtis CA, Ashwood ER, eds. Tietz fundamentals of clinical chemistry, 4th ed. Philadelphia: WB Saunders; 1996. 283–335.
12. Winn-Deen ES, David H, Sigler G, Chavez R. Development of a direct assay for α-amylase. Clin Chem 1988;34:2005–2008.
13. Vissers RJ, Abu-Laban RB, McHugh DF. Amylase and lipase in the emergency department evaluation of acute pancreatitis. J Emerg Med 1999;17:1027–1037.
14. Zakowski JJ, Bruns DE. Biochemistry of human alpha amylase isoenzymes. [Review] Crit Rev Clin Lab Sci 1985;21:283–322.
15. Mifflin TE, Hortin G, Bruns DE. Electrophoretic assays of amylase isoenzymes and isoforms. Clin Lab Med 1986;6:583–599.
16. Rinderknecht H, Wilding P, Haverback BJ. A new method for the determination of alpha-amylase. Experientia 1967;23:805.
17. Nishide T, Emi M, Nakamura Y, Matsubara K. Corrected sequences of cDNAs for human salivary and pancreatic alpha-amylases.Gene. 1984; 28:263–270.
18. Rosenblum JL, Hortin GL, Smith CH, Pashos GE, Landt M. Macroamylases: differences in activity against various-size substrates. Clin Chem 1992;38:1454–1458.
19. Tarvers RC, Church FC. Use of high-performance size-exclusion chromatography to measure protein molecular weight and hydrodynamic radius. An investigation of the properties of the TSK 3000 SW column. Int J Pept Protein Res 1985;26:539–549.
20. Harris JM. Introduction to biotechnical and biomedical applications of poly(ethylene glycol). In: Harris JM, ed. Poly(ethylene glycol) chemistry, New York: Plenum Press; 1992. 1–14.
21. Squire PG. Hydrodynamic characterization of random coil polymers by size exclusion chromatography. Methods Enzymol 1985;117:142–153.
22. David H. Hydrolysis by human α-amylase of p-nitrophenyl oligosaccharides containing four to seven glucose units. Clin Chem 1982;28: 1485–1489.
23. Brayer GD, Sidhu G, Maurus R, et al. Subsite mapping of the human pancreatic α-amylase active site through structural, kinetic, and mutagenesis techniques. Biochemistry 2000;39:4778–4791.