A Monoclonal Antibody That Specifically Inhibits Human Salivary α-Amylase

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Our monoclonal antibody 88E8 specifically binds to and inhibits human salivary α-amylase (EC 3.2.1.1) and cross reacts negligibly with the pancreatic isoenzyme, inhibiting it by <1%, as compared with about 90% for the salivary isoenzyme. The antibody binds the S1 and S2 types of salivary α-amylase, but no pancreatic α-amylase isoenzyme forms. A pancreatic α-amylase assay involving 88E8 is under development, with α-glucosidase as auxiliary enzyme and p-nitrophenyl-maltoheptaoside as substrate; we give preliminary data on this assay. The assay has to be done by substrate start, because the antibody interacts very slowly with the enzyme in the presence of substrate. Assay results for pancreatic α-amylase correlate well with those for isoamylase assayed with use of an inhibitor from wheat-germ.

Additional Keyphrases: isoenzymes • enzymatic methods

Two isoenzymes of human α-amylase (EC 3.2.1.1) are described in the literature, the salivary α-amylase and the pancreatic isoenzyme, which differ by 7% in their amino acid sequence (1, 2). Owing to post-translational modifications, these isoenzymes give rise to several multiple forms, which can be distinguished by their electrophoretic mobility (3). Assay of the activity of total α-amylase in human serum, especially of the activity of the pancreatic isoenzymes, is important for the diagnosis of acute pancreatitis (4). The isoenzymes can be distinguished from each other by electrophoresis on cellulose acetate (5), isoelectric focusing (6), or selective inhibition of the salivary isoenzyme by wheat-germ proteins (7, 8). This inhibition is incomplete; hence, a standard curve is needed in performing the assay. Seeking a more convenient assay for the pancreatic isoenzyme, we searched for monoclonal antibodies (MAB) that could discriminate between the two isoenzymes. As a first result, we found an antibody that selectively binds to the salivary isoenzymes (9). Also, this antibody could be immobilized to a matrix, thus allowing selective removal of the salivary α-amylase from the serum specimen (10). Independently, other groups found antibodies of the same specificity (11, 12).

Here we report on a monoclonal antibody that specifically inhibits the salivary isoenzyme without any detectable cross reactivity against pancreatic α-amylase. Preliminary data on the analytical performance of this antibody under amylase assay conditions are shown. An isoamylase assay specific for the pancreatic amylase is under development. Its characteristics will be described in a forthcoming publication.

Materials and Methods

Materials

The human α-amylase solutions were the same as described elsewhere (9).

The equipment for isoelectric focusing in polyacrylamide gels was from LKB, Bromma, Sweden. The chemicals for isoelectric focusing were from Serva, Heidelberg, F.R.G. We used a Chromoscan 3 (Joyce Loel, Gateshead, England) to record the densitometric tracings of the gels.

We determined the activity concentration of α-amylase kinetically at 25 °C by the p-nitrophenyl-maltoheptaoside (substrate) method (cat. no. 568589; Boehringer Mannheim GmbH), using either a conventional spectrophotometer (Uvicron 610CL; Kontron Instruments GmbH, D-8067 Eching, F.R.G.) or the mechanical analyzer Cobas Fara (Hoffmann-La Roche & Co. AG, Basel, Switzerland). The activity of human pancreatic α-amylase was determined at 25 °C with the wheat-germ inhibitor method from Boehringer Mannheim GmbH (cat. no. 779610) with the mechanical analyzer Cobas Fara, as described by the manufacturer of the test kit.

Isoelectric Focusing of Human α-Amylase

For the isoelectric focusing of human α-amylases on polyacrylamide gels we used 10 × 21 cm gels, 300 μm thick, containing 51 g of acrylamide, 2.6 g of bisacrylamide, 50 g of glycerin, 25 mL of Servalyt 4–9, 50 mL of Servalyt 6–7, and 1.2 g of (NH₄)₂S₂O₈ (as polymerization initiator) per liter of water. The gels were cooled to 4 °C; the electrode wicks were soaked in 0.1 mol/L solution of NaOH (cathode) or H₃PO₄ (anode) and were placed directly on the surface of the gels. Before the 10-μL sample was applied, the gel was prefocused for 1 h at 500 V. Activity concentrations of the samples applied ranged from 150 to 600 U/L. The gels were focused for 18 h at 800 V, then for 2 h at 1200 V. We then overlaid the gel with a 0.5-mm-thick substrate gel as described earlier (9) and incubated for 2 h at 37 °C. The α-amylase activity appeared on the polyacrylamide gel as blue bands on a transparent background. The gels were dried at room temperature for about 18 h, and the densities of the bands were recorded at 624 nm.

Preparation of the Monoclonal Antibodies

Immunogen: The immunogen was prepared as follows. Mix 1.6 mL of human salivary α-amylase [3.3 g of enzyme per liter of Tris buffer (50 mmol/L, 1 mmol of CaCl₂ per liter, pH 8.0)] with 10 μL of tetraniotromethane (100 g per liter of ethanol) and incubate for 12 h at room temperature. Then dialyze the modified α-amylase twice against the CaCl₂-containing Tris-buffer to remove nonreacted tetraniotromethane.

Spectrophotometric assay at 350 nm according to Sokolovsky et al. (13) indicated that 2.6 nitro groups were introduced per α-amylase molecule.

Immunization, fusion, and culture conditions are the same as described earlier (9), except that the myeloma cell line X63Ag6.53 was used for fusion, and two cultures of each fusion were selected and cloned. All further steps of preparation and purification of the antibody were as described earlier (9).

Screening: To screen for monoclonal antibodies that spe-
cifically inhibit human salivary α-amylase, we developed an immunoassay as follows. Pipet into 96-well microtiter plates (Nunc, Roskilde, Denmark) 50 μL of either human salivary or human pancreatic α-amylase with an activity concentration of 400 U/L in phosphate buffer (50 mmol/L, pH 7.0) containing 1 g of bovine serum albumin and 50 mmol of NaCl per liter. Add 50 μL of cell-culture supernatants containing monoclonal antibody against α-amylase, or only culture medium as a control probe, either to the salivary or to the pancreatic α-amylase wells. Seal the plates with a plate sealer (Dynatech Deutschland GmbH, Denkendorf, F.R.G.), shake for 5 min at room temperature on a shaker (IKA MTS4; Janke & Kunkel GmbH and Co. KG, D-7813 Staufen, F.R.G.), and then incubate them for 25 min at 30°C. Add 50 μL of a threefold concentrated α-amylase reagent (cat. no. 568589; see Materials) containing p-nitrophenyl-maltoheptaoside as substrate and α-glucosidase as auxiliary enzyme. After shaking, develop the plates for 20 min at 37°C, and then measure the yellow color at 405 nm (we used a SLT Microplate Reader, SLT-Lab Instruments Deutschland GmbH, D-5063 Overath, F.R.G.). Wells that contain antibodies inhibiting either salivary or pancreatic α-amylase, or both, show about 10% of the color intensities of the control probes (containing antibody-free culture medium).

Immunoprecipitation of Salivary α-Amylase in Human Serum

Formation of the immunoprecipitant as well as adsorption of the salivary enzyme to it was performed as described earlier (9). For antibody 88E8, we used 40 μL of a solution having a concentration of 15.4 g/L.

Inhibition Studies

Preparation of Fab fragments: The purified monoclonal antibody 88E8 was split to its Fab fragments by the method of Nisonoff (14).

Determination of Kᵢ-values: Dissolve the purified monoclonal antibody 88E8 in phosphate buffer (50 mmol/L, pH 7.0) containing 50 mmol of NaCl per liter of buffer. Mix 50 μL of different concentrations of this antibody solution with 50 μL of human saliva α-amylase solution (about 500 U/L) and incubate for 1 h at room temperature. Mix 1 mL of substrate solution containing 30 U of α-glucosidase (EC 3.2.1.20) and different amounts of p-nitrophenyl-maltoheptaoside in phosphate buffer (50 mmol/L, NaCl 30 mmol/L, pH 7.1) with 50 μL of the α-amylase-antibody solution. Determine the initial reaction velocity at 25°C by recording the absorbance at 405 nm. Plot the kinetics according to Lineweaver and Burk (15). We analyzed the inhibition pattern as described by Segel (16).

Inhibition curves: Antibody 88E8 was incubated for 1 h at room temperature with either human salivary or human pancreatic α-amylase in phosphate-buffered isotonic saline containing 1 g of bovine serum albumin per liter. Residual α-amylase activity was determined kinetically at 25°C with the p-nitrophenyl-maltoheptaoside method by serum start with the mechanical analyzer Cobas Fara. Residual activity was calculated as the percentage of the activity of the noninhibited α-amylase.

Preliminary Pancreatic-α-Amylase Test Kit

The final reaction mixture concentrations of the reagents in the pancreatic α-amylase test kit are: 30 000 U of α-glucosidase, 5 mmol of p-nitrophenyl-maltoheptaoside, 50 mmol of NaCl, and 30 mg of antibody 88E8 per liter of phosphate buffer (50 mmol/L, pH 7.1).

The test procedure is as follows. Mix 20 parts of reagent mixture without substrate for 5 min with one part of the sample, at 25°C. Then start the reaction by adding two parts of substrate solution. Determine the reaction velocity at 405 nm between minutes 4 and 7. Use a factor of 7667 to calculate the residual activity (R). Determine total α-amylase activity of the sample (T) by the same procedure but omit monoclonal antibody 88E8 from the reagent mixture. Pure human salivary α-amylase will be inhibited by 91%; pure human pancreatic α-amylase is not inhibited. Therefore from total and residual activity calculate the pancreatic α-amylase activity concentration (P) by using the following equation:

\[ P = \frac{[100/(100 - 9)] \times R - [9/(100 - 9)] \times T}{1.1 \times R - 0.10 \times T} \]

Calculate the true salivary α-amylase activity concentration (S) of the sample from the difference between total and pancreatic α-amylase activity concentration: \( S = T - P \).

Results

Salivary α-Amylase-Inhibiting Monoclonal Antibody

The monoclonal antibody 88E8 specifically binds and inhibits human salivary α-amylase when used as described above. It does not bind to or inhibit the human pancreatic α-amylase isoenzyme. The antibody has an IgG subtype 2a, as determined by the method of Storch and Lohmann-Matthes (17). Figure 1 shows the isoelectric focusing of either pure human salivary or pancreatic α-amylase or of human serum, which were pretreated with monoclonal antibody precipitates. Antibody 88E8, described here, and the antibody 66C7, which only binds specifically to the human salivary α-amylase (9), were used. Both antibodies bind to the salivary α-amylase isoenzymes. Both isoenzyme forms, S1 and S2, were removed from the sera. Figure 2 shows the densitometric tracings of one serum before (lane I) and after treatment with the antibody 88E8 (lane II). The S2 isoenzyme band disappears nearly completely, as does most of the S1 isoenzyme band. P2 and the other pancreatic α-amylase bands seem not to be influenced by the antibody treatment. In Figure 2, lane II also shows the P4 isoenzyme form, which could not be seen before the antibody treatment (lane I) because of the high activity of the S1 isoenzyme.

Inhibition Studies

Figure 3 shows the inhibition pattern of 88E8 with the substrate p-nitrophenyl-maltoheptaoside in the Lineweaver–Burk illustration. The pattern indicates a mixed-type noncompetitive inhibition (15) of the enzyme salivary α-amylase by 88E8 (Figure 4), which means that the antibody can either bind to the free enzyme (E) or to the enzyme–substrate complex (E·S). Both substrate complexes (E·S) and (E·MAB·S) are reactive. Table 1 shows the inhibition constants of the antibody 88E8. As can be seen, it makes no significant difference in the inhibition pattern whether the antibody is used as total IgG or as a Fab fragment.

Figure 5 shows the percentage residual activity of human pancreatic and salivary α-amylase after incubation with 88E8 as a function of the final antibody concentration. At an antibody concentration of about 5 mg/L, there is about 90% inhibition of the salivary α-amylase. Table 2 shows that 88E8, either as IgG or Fab fragment, shows the same
Inhibition power (Fab = 18 mg/L has about the same normality as IgG = 30 mg/L). The salivary α-amylase is not completely inhibited, even with a final 88E8 concentration of 300 mg/L.

Pancreatic α-Amylase Test Description

Figure 6 shows A/t diagrams, under the conditions detailed in the legend, for the action of pancreatic α-amylase and total α-amylase test kits on a sample containing salivary α-amylase. The binding velocity of 88E8 to human salivary α-amylase evidently is strongly decreased when the substrate p-nitrophenyl-maltoheptaoside is present (compare line 2 with line 3).

Figure 7 shows a method comparison for pancreatic α-amylase determination at 25 °C using a wheat-germ inhibitor test kit (x) and the 88E8 test kit (y). The final concentrations of reagents in the latter kit are as detailed above, except the buffer concentration is 100 mmol/L. The correlation between results of the two tests is very good. The slope,

Fig. 3. Inhibition pattern of human salivary α-amylase treated with different concentrations of the monoclonal antibody 88E8

Fig. 4. Reaction scheme of human salivary α-amylase (E), the substrate p-nitrophenyl-maltoheptaoside (S), and antibody 88E8 (MAB, either IgG or Fab fragments)

P = products

Table 1. Kinetic Constants* of Antibody 88E8 as IgG or Fab Fragment, with Human Salivary α-amylase and p-Nitrophenyl-maltoheptaoside as Substrate*

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>αK0</th>
<th>αKp</th>
<th>Ks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fab</td>
<td>1.8</td>
<td>9.6</td>
<td>2.2</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*According to Fig. 3.
* Determined at 25 °C in phosphate buffer (100 mmol/L, pH 7.1) containing 50 mmol of NaCl and about 30 000 U of α-glucosidase per liter.
Fig. 5. Inhibition of human salivary and pancreatic α-amylase by the monoclonal antibody 88E8
88E8 and α-amylase were preincubated at room temperature for 1 h, then the α-
amylase activity was determined at 25 °C by the serum-start method.

Table 2. Inhibition of Human Salivary and Pancreatic α-Amylase by the Antibody 88E8

<table>
<thead>
<tr>
<th>Form and concn of antibody 88E8 added</th>
<th>Pancreatic α-amylase</th>
<th>Salivary α-amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/L</td>
<td>%</td>
</tr>
<tr>
<td>No antibody</td>
<td>1332</td>
<td>100</td>
</tr>
<tr>
<td>IgG, 30 mg/L</td>
<td>1341</td>
<td>101</td>
</tr>
<tr>
<td>IgG, 300 mg/L</td>
<td>1301</td>
<td>98</td>
</tr>
<tr>
<td>Fab, 18 mg/L</td>
<td>1330</td>
<td>100</td>
</tr>
<tr>
<td>Fab, 180 mg/L</td>
<td>1322</td>
<td>99</td>
</tr>
</tbody>
</table>

α-Amylase activity was determined kinetically at 25 °C by serum start.

Pancreatic α-amylase activity was determined by comparing the residual activity of the sample with and without antibody treatment.

Table 2 shows the inhibition of human salivary and pancreatic α-amylase by the antibody 88E8.

Discussion

The monoclonal antibody 88E8 is the first antibody that specifically binds to and inhibits human salivary α-amylase, showing a cross reactivity to human pancreatic α-amylase of less than 1%. The earlier-described salivary α-amylase-specific monoclonal antibodies (9, 11, 12) only allow the determination of isoamylase by a separation step, because these antibodies bind only to human salivary α-amylase; in

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inhibitor test and with enzyme immunoassay tests involving monoclonal antibody MAB 66C7. A test useful for the routine determination of pancreatic α-amylase in human serum and based on the inhibition by antibody 88E8 is under development. A detailed description of this assay, including its performance at 25, 30, and 37 °C in the normal and pathological range, will be published.

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References