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Characterization of a Monoclonal Antibody that Specifically Inhibits Pullulanase Activity of *Bacillus circulans* Amylase-Pullulanase Enzyme

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ABSTRACT

A monoclonal antibody (MAb) against amylase-pullulanase enzyme from *Bacillus circulans*, which hydrolyzes not only the α-1,6-glycosidic linkage but also the α-1,4-glycosidic linkage to the same extent, has been produced by the fusion of BALB/c mouse spleen cells immunized with the native enzyme and P3x63Ag8U1 myeloma cells, and examined for inhibition of pullulanase activity in order to characterize the catalytic site of the pullulanase. The MAb recognizes active enzyme, but not the SDS-denatured or heat-inactivated protein, indicating that the antibody is highly conformational-dependent, specific for active enzyme. The antibody inhibited the pullulanase activity, but not amylase activity. The monoclonal antibody immunoblotted the enzyme and immunoprecipitated the enzyme. The immunoprecipitation was inhibited in the presence of substrate, pullulan, and the MAb competitively inhibited the binding of pullulan to the enzyme. The MAb, therefore, recognizes the pullulan-binding site of the enzyme. Kinetic analysis showed that the MAb inhibited pullulanase activity with inhibition constant (K_i) of 0.77 μg/mL, providing evidence that the antibody decreases the catalytic rate of enzyme activity and has an effect on substrate binding. These results strongly confirm the previous observations that APE may have two different active sites responsible for the expression of amylase and pullulanase activities (Kim, C. H. and Kim, Y. S. *Eur. J. Biochem.* 1995, **227**, 687–693).

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Abbreviations: APE, amylase-pullulanase enzyme; MAb, monoclonal antibody; P3U1, P3x63Ag8U1, ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HRPO, Horse radish peroxidase; BCIP, 5-bromo-4-chloro-3-indolyl- phosphate; NBT, nitro blue tetrazolium, DNS, dinitrosalicylic acid.

INTRODUCTION

A novel amylase-pullulanase enzyme (APE) which has been isolated, purified and characterized from the culture broth of *B. circulans*, functions as α-1,4- and α-1,6-glucanohydrolase *(1,2)*. The enzyme, therefore, belongs to the class of enzymes called bifunctional hydrolase. However, it is not clear whether a single site on the enzyme is responsible for the expression of both activities or not, and little information as to the structure of the bifunctional enzyme has been reported *(3)*. However, the kinetic studies showed that this enzyme seemed to have two independent active sites on one polypeptide *(3)*.

In general, the structures of enzymes and those of their substrate-binding sites have been studied by X-ray crystallography *(4,5)* or subsite-labeling method *(6,7)*. Since it has been generally accepted that an emerging and potentially useful method for the study of enzymes involves antigen–antibody binding reaction, the hybridoma technique has been effectively used in many biochemical investigations *(8)*. Monoclonal antibodies (MAbs) are used widely for the detection, estimation, and purification of specific proteins: the unique specificity of MAbs for single antigenic determinants makes them extremely powerful reagents for studying the relationship between the structure and function of many macromolecules such as proteins, especially enzymes. For such investigations it is often desirable to generate a variety of antibodies that react with different sites and/or conformations of a given antigen, such as proteins, including enzymes *(9)*. Once MAbs specific for an enzyme are raised by the technique, the MAbs can be used as powerful tools to study structural and functional domains of the enzyme. Therefore, we attempted to prepare MAbs against APE in order to locate and characterize the catalytic sites of the enzyme. We have used a previously developed screening assay, a gel-based direct method *(9)*, to select MAbs that selectively inhibit pullulanase activity of APE.

We presently describe the preparation of a MAb-directed against APE, which selectively inhibits pullulanase activity in APE, and evidence that the MAb is specific for the pullulan-binding site of the enzyme. In addition, the MAb seems to recognize only the active enzyme, but not the denatured form.

MATERIALS AND METHODS

Materials

BALB/c mice (female, 4 wk old) were used. Amylase-pullulanase enzyme of *B. circulans* F-2 was purified as described previously *(2)*. Pullulan (M_r = 65,000) and reactive red 120 were purchased from Sigma Chemical (St. Louis, MO). The next materials used were obtained from the sources indicated: *Staphylococcus aureus* cells (Calbiochem, Richmond, CA), mouse monoclonal antibody isotyping kit (Bio-Rad Laboratories, Hercules, CA), prepacked columns of immobilized protein-A (Bio-Rad), mouse IgG (Promega, Madison, WI), and peroxidase-conjugated F(ab')2 fragment goat antimouse IgG (Promega). The soluble starch from E. Merck was reduced with sodium borohydride and used as a substrate on amylase assay. Other reagents necessary for the preparation of MAbs were purchased from the sources mentioned in a previous report *(9)*. All other biochemicals were purchased from Wako Pure Chemicals (Tokyo, Japan) and Sigma.

Immunization, Fusion, and Preparation of MAbs

The APE used for immunization and screening of MAbs by enzyme-linked immunosorbent assay (ELISA) was purified from bacterial culture as described previously *(2)*. BALB/c mice (4 wk old) were immunized intraperitoneally with 200 μg of purified APE in Freund's complete adjuvant. After a week they received a second injection of 200 μg of enzyme in an Freund's incomplete adjuvant. Four days after the third injection, the spleen was aseptically removed and gently dissected to separate cells from stroma. Spleen cells were fused with myeloma cells P3U1 (abbreviated from P3-X63-Ag8-U1), using 50% polyethylene-glycol 4000 as a fusion agent according to the standard techniques *(10)*. Hybridoma cells were grown in hypoxanthine-aminopterin-thymidine selection medium containing 15% fetal bovine serum on wells of 96-well microplates (Becton Dickinson, NJ). After 9 d, the supernatants of the cultures were tested for the production of MAbs against the enzyme using ELISA described below.

To produce large amounts of antibodies in vitro, hybridomas originating from single cells were grown at a high density in 75 cm^2 flasks (working volume of 30 mL) until more than 95% of the cells were dead as determined by a trypan blue exclusion assay. In addition, high titer ascites fluids were collected from Pristane-primed *(11)* BALB/c mice that had been given as ip injection of 2–10 × 10^6 viable cultured cells. In both cases the cells and debris were removed by centrifugation and the preparations were stored at –20°C. Ascites fluids routinely contained 3–40 mg/mL IgG, which was 200- to 1000-fold greater than the level achieved in tissue culture. Antibodies were purified by ammonium sulfate fractionation (30–60% saturation) following Protein-A Sepharose chromatography using Affi-Gel Protein A MAPS-II kit (Bio-Rad Laboratories). Isotype was determined

using a peroxidase labeling immunoassay kit (Bio-Rad Laboratories) according to the procedure recommended by the manufacturer.

ELISA Screening of MAbs

The screening of MAbs to antigens was measured by ELISA as follows: To each well of a 96-well plate (Nunclon, Intermed, Denmark) was added fifty microliters of phosphate-buffered saline (PBS) containing 0.5 μg pure APE and allowed to bind overnight at 4°C. Following the removal of the binding solution, which could be recycled, the plates were rinsed five times with distilled water and flooded with a solution containing 10 m*M* Tris-HCl (pH 7.8), 150 m*M* NaCl, 0.3% bovine serum albumin (BSA), and 0.3% skim milk (blocking buffer) to saturate protein binding sites and then incubated for 1 h at room temperature. The solution was shaken out, and the plates were rinsed in water and drained. At this time the culture supernatant (usually 50 μL) was added to the wells, and the plates were incubated at room temperature for 2 h. The plates were then washed three times with 0.1% Tween-20 in PBS. Goat anti-mouse IgG conjugated to peroxidase (Promega) was diluted 1:10,000 with blocking buffer and 50 μL of this antibody was added to each well. After incubation for 1 h at room temperature, the plates were washed with 0.1% Tween-20 in PBS. Finally, the wells were incubated with 100 μL/well of a solution containing 1*M* diethanolamine-HCl (pH 9.6), 1 m*M* $MgCl_2$ and 0.2 mg/mL of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Promega) and nitro blue tetrazolium (NBT, Promega) *(9)*. The plates were incubated at 30°C and positive wells were observed by the blue-brown colors. Cells secreting antibodies specific to APE were cloned with feeder cells by the limiting dilution method.

Polyacrylamide Gel Electrophoresis (PAGE) and Immunological Blotting Analysis

PAGE was carried out at 25°C with a constant current of 20 mA in a 12% polyacrylamide slab gel (1-mm thickness) at pH 8.3 using the discontinuous buffer system of Brewer and Ashworth *(12)*. Following electrophoresis, the gel was cut into halves. While one half was examined for immunoblotting analysis, the other half of the gel was tested for inhibition assay of amylase or pullulanase activity with selected MAbs. For screening of MAbs inhibiting at least one of both enzymatic activities, each lane of electrophoresed gel was cut and separately treated with MAbs as described previously *(9)*.

Immunoblotting analysis was done as described by Thorpe et al. *(10)*. After APE of *B. circulans* F-2 was resolved on polyacrylamide slab gels, the protein bands were transferred electrophoretically to the nitrocellulose membranes (Bio-Rad) and each lane of membranes was cut. These membranes were separately incubated with each MAb. The bound MAbs were detected by a sandwich assay with biotinylated antimouse IgG followed by

avidin-labeled HRPO and color was developed using 4-chloro-1-naphtol as a substrate.

Selection of MAb Inhibits Pullulanase Activity by the Gel-Based Direct Screening

After electrophoresis of the APE, slab gel containing APE band was sliced in each lane. The sliced gel was individually incubated with each culture supernatant (usually 2.5 mL) of hybridoma that produce MAbs able to bind to APE at room temperature for 2 h. The gels were then washed with PBS and tested for zymogram of amylase and pullulanase activity *(8,13)*. All electrophoresis were performed in parallel, one for amylase and the other for pullulanase activities.

Zymogram of amylase or pullulanase activity was obtained as follows. For amylase activity staining, the sliced gels were incubated at 30°C for 30 min in 50 m*M* sodium phosphate buffer containing 2% soluble starch, and the enzymatic activity on the gel was detected by 0.2% I_2/KI solution *(14)*. Amylase activity showed a clear band on blue background. For the pullulanase zymogram, pullulan-reactive red was prepared by the procedure of Rinderknecht et al. *(15)*, with the dyestuff covalently bound to pullulan. The pullulan-reactive red agar plate was cast by pouring 15 mL of a heated solution (60°C) of pullulan-reactive red and agar at 2.5 and 2.1% (w/v), respectively, in 100 m*M* sodium phosphate buffer (pH 7.0) into an immunodiffusion plate (45 × 95 mm) (Naperville, IL). The polyacrylamide gel was soaked twice in fresh 25% isopropanol and 50 m*M* phosphate buffer (pH 7.0) for 10 min. The gel was then immersed in fresh phosphate buffer without isopropanol for 10 min and then repeated in fresh buffer. The gel was blotted dry and overlaid on top of the pullulan-reactive red agar plate. The plate was covered with a lid, tightly sealed with parafilm, and incubated at 30°C for 2 h. The active pullulanase appeared as a clear band on a red background.

Immunoprecipitation

The APE (0.5 μg) of APE was incubated with varying amounts of the MAb in 175μL of 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.02% BSA in an ice bath for 2 h, after which 25 μL of a 12% (w/v) cell suspension of *Staphylococcus aureus* in 20 m*M* phosphate buffer was added to the mixtures. After a 1-h incubation in an ice-bath, the mixtures were centrifuged at 1600*g* for 15 min. The remaining enzyme activities in the supernatants were assayed as described below. For the experiments investigating the effect of pullulan on the immunoprecipitation, the enzyme was preincubated with 50 mg of pullulan for 25 min in an ice bath, after which it was incubated with the MAb. The other conditions were the same as described above. All the experiments with pullulan were carried out in ice cold for prevention of its hydrolysis.

Enzyme Assay and Inhibition by Purified Antibody

Amylase and pullulanase activities were measured in a 200 μL reaction mixture containing 100 μL of 2% reduced soluble starch and 100 μL pullulan with a 100μL of enzyme solution in 20 m*M* sodium phosphate buffer (pH 7.0), respectively. The reaction was carried out for 30 min at 30°C and the reducing sugar produced was determined by the dinitrosalicylic acid (DNS) method *(16)* using glucose as a standard. One unit of amylase or pullulanase activity is defined as the amount of enzyme which produces one μmol of reducing sugar per minute.

To see the inhibitory effect of MAb on pullulanase activity in APE, the purified APE (10 μg) in a total volume of 0.5 mL in 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.2% BSA was preincubated at 4°C for 30 min with varying amounts of the MAb. The reaction mixtures were further incubated with 0.5 mL of solution containing 2% reduced soluble starch and 2% pullulan at 30°C for 30 min. After incubation, the residual enzyme activities in the reaction mixtures were assayed as described above by DNS method (16). The values represent the percentages of the residual activities to the enzyme activities determined in the absence of any antibody.

RESULTS AND DISCUSSION

When mice were immunized with denatured APE as an antigen, the antisera raised against the enzyme showed only low antibody titers. The fusion of the spleen cells from the mice with P3U1 myeloma cells resulted in the production of a small number of hybridoma cells producing MAbs against the enzyme, and the class of the MAbs produced by the cloned hybridomas was only IgM. However, the immunization with the native enzyme resulted in the production of high antibody titers of the antisera against the enzyme. Therefore, we used the native enzyme as an antigen in the present study. These results are in contrast to those of a MAb recognizing the FAD-binding site of 4-amino benzoate hydroxylase from *Agaricus bisporus (17)*. The production of MAb, which can selectively inhibit at least one of two enzyme activities, is infrequent, although the mechanism(s) of enzyme inhibition or the epitope(s) remain unknown. This result was similar to other cases of enzyme-inhibiting MAbs *(18,19)*. One probable reason may be the characteristics of the immune system to respond to the foreign antigen because the enzyme may have a variety of antigen determinants. When the spleen cells obtained by immunization with the native enzyme were fused with P3U1 myeloma cells, a large number of hybridoma cells were detected by the first screening test with ELISA. The positive hybridoma cells were cloned three times by limiting dilution method. Culture supernatant containing the MAbs specific to APE was flooded on the sliced gel that contained the electrophoresed active APE bands, following amylase or pullulanase activity staining. MAbs inhibiting the pullu-

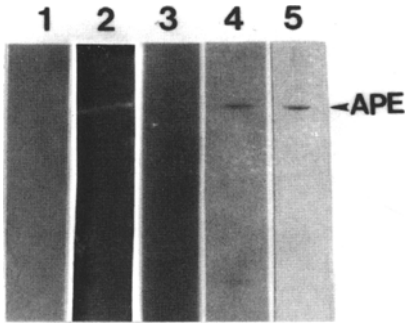

Fig. 1. Zymogram and immunoblot analysis of APE. APE (0.5 μg per lane) was electrophoresed on 12% native PAGE and then two lanes were subjected to direct inhibition assay with pullulan (lane 1) and soluble starch (lane 2). For immunoblot analysis, the remaining gel was transferred to nitrocellulose membranes and either stained with Amido Balck 10B (lane 5) or immunoblotted with the MAb (0.7 μg/mL lane 4). Lane 3, immunoblot analysis of the isolated MAb following SDS-PAGE. For activity inhibition using zymogram, excess amount of MAb (20 μg) were overlayed on the gel at 4°C for 1 h, and then treated with 2% Iodine-KI solution or with pullulan-reactive red agar plate. Arrowhead denotes corresponding APE position.

lanase activity only were selected on the native gel. Finally, one hybridoma cell line was established. The class of the MAb was IgGl having γ_d heavy chain and λ light chain.

A large amount of the antibody was reproduced, purified to see the inhibitory kinetic of the MAb against amylase and pullulanase activities in detail, and the APE was preincubated with varying amounts of the antibody. The MAb inhibited about 65% of the pullulanase activity (showing 35% as residual activity) even with 1.0 μg of the antibody, whereas the amylase activity was scarcely inhibited. When using zymogram assay, the MAb did not inhibit catalytic activity of the amylase (Fig. 1, lane 2), while selectively inhibiting the pullulanase activity (Fig. 1, lane 1). This result clearly showed that the MAb strongly inhibited only pullulanase activity, but not amylase activity.

On the other hand, the MAb immunoblotted APE on a nitrocellulose paper transferred from a polyacrylamide gel after native PAGE (Fig. 1, lane 4), whereas the MAb did not immunoblot APE from a SDS-PAGE (Fig. 1, lane 3). The binding of APE to MAb was also monitored by a ELISA. In this assay APE was immobilized on plates, and the amount of MAb bound on the solid phase was quantified with a biotin-labeled second antibody. We analyzed the binding of APE, which had been submitted to heat denaturation at various temperatures for 30 min (Fig. 2). I found that pullulanase activity completely disappeared at 80°C. This thermal denaturation is accompanied by a transient increase followed by a subsequent reduction of the immunoreactivity. This effect may result from a variation of the affinity of the immobilized enzyme. The signal was

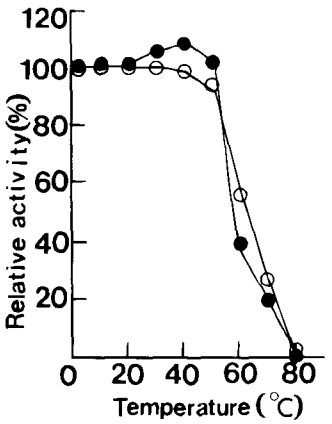

Fig. 2. Effect of thermal denaturation on the reactivity of APE with MAb. The enzyme was preincubated in McIlvaine buffer (pH 6.5) at various temperatures for 30 min. For the remaining activity, the half of the enzyme solution was assayed in the standard assay condition. The remaining half of the solution were coated on a 96-well plate and reactivity of APE with MAb was measured by ELISA. ○, enzyme activity; ●, ELISA at 492nm using MAb. All values are normalized at 100% at 20°C.

totally abolished when the MAb was used, exactly in parallel with the loss of catalytic activity, demonstrating that this antibody recognized only the native form of the enzyme. This result suggests that the MAb recognizes a conformational epitope on the enzyme. This showed that the MAb does not bind detergent-denatured or thermal-inactivated APE, indicating that the MAb retained only active molecules. The present results confirm that the MAb is sensitive to the conformation of active APE, since it has no affinity for denatured protein. It would be extremely interesting to localize the region of the enzyme that binds the MAb, since this might give us some information on the difference between active and inactive molecules. Unfortunately, this can not be determined by classical epitope-mapping methods since only the complete, correctly folded polypeptide chain is likely to be recognized by this antibody. The present results, therefore, establish that the MAb is highly conformation-dependent, and recognizes only active APE molecules.

On the other hand, Fig. 3 demonstrates that the MAb immunoprecipitated the enzyme and that the immunoprecipitation was inhibited in the presence of pullulan. These results indicate two possibilities: the one is that the MAb binds to the substrate-binding site of the enzyme. The other is that the MAb binds to the site different from the substrate-binding site that would be buried in the interior part of the protein by the binding of substrate to the enzyme so that the MAb could not bind to the enzyme. Thus, the effect of the MAb on the binding of substrate to the enzyme was examined. The kinetic constant for the hydrolysis of pullulan was calculated from the measurements of the amount of reduced sugar (as maltotriose) released. The K_i value for pullulan (average M_r 65000) was obtained at

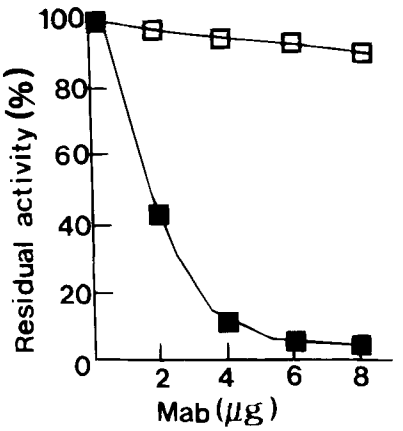

Fig. 3. Immunuprecipitation of APE with the MAb in the absence (□) and presence (■) of pullulan. The purified APE (0.5 μg) was incubated with varying amounts of the MAb in 20 m*M* sodium phosphate buffer (pH 6.5) containing 0.02% BSA in a total volume of 175 μL, after which the complexes were incubated and precipitated with a 12% (w/v) cell suspension of *Staphylococcus aureus*, as described in Material and Methods. In order to investigate the effect of pullulan on the immunoprecipitation, the enzyme (0.5 μg) was preincubated with 50 mg of pullulan in 175 μL of 20 m*M* phosphate buffer containing 0.02% BSA, after which the bound enzyme reconstituted was subjected to the above-mentioned immunoprecipitation reaction. The values represent the percentages of the residual activities in the supernatants to the enzyme activity assayed in the absence of any antibody.

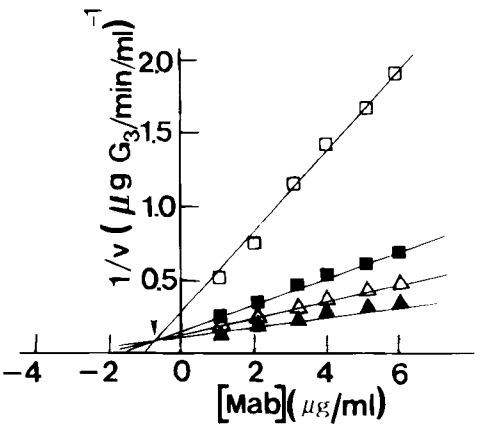

Fig. 4. Effect of the MAb on the binding of pullulan to the enzyme. Final pullulan concentrations of 0.2 mg/mL (□), 0.6 mg/mL (■), 1.2 mg/mL (△), and 2.4 mg/mL (▲) were used.

substrate concentrations between 0.125–1.0 mg/mL at 0.2-mg/mL increments. Kinetic analysis clearly showed that the Mab competes with pullulan for binding to the enzyme and the Mab inhibited pullulanase activity with substrate inhibition constant (K_i) pf 0.77 μg/mL (Fig. 4). The value reflects the affinity of the MAb for APE. These findings demonstrate that

the MAb binds to the substrate-binding site of the enzyme. Inhibition of pullulanase activity in the APE enzyme suggests a possibility that the MAb competitively interacts with the substrate-binding site. This is, therefore, the first report on the preparation of MAbs specific for the substrate-binding site of pullulanase. The isolation and characterization of the pullulan-binding site of APE are in progress using MAb and gene for APE.

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