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MONOCLONAL ANTIBODIES TO YEAST CATALASE T

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SUMMARY: Hybridoma cell lines secreting antibodies directed against Saccharomyces cerevisiae catalase T were constructed by fusing spleen cells of mice immunized with catalase T with P3x63Ag8 mouse myeloma cells. Culture supernatants were assayed for specific antibodies by incubation with ^{35}S-labelled yeast extracts, adsorption of the immune complexes to Protein A - carrying Staphylococus aureus cells and analysis of the adsorbed yeast proteins by sodium dodecylsulfate gel electrophoresis. Two hybrid clones were isolated mediating adsorption of a protein with electrophoretic mobility of catalase T; one of them, showing considerably higher activity, was characterized further. Antibodies produced by this clone belong to the IgG class of immunoglobulins; they can be used for immunoadsorption, but not for direct immunoprecipitation and recognize authentic catalase T as well as catalase T apoprotein.

In the course of studies on the regulation of biosynthesis of yeast catalase (1), we have attempted to isolate monoclonal antibodies (2-4) specific for catalase T of Saccharomyces cerevisiae. This enzyme (E.C. 1.11.1.6) is a tetrameric hemoprotein of molecular weight 240 000 consisting of 4 identical subunits (5). This paper describes the isolation and characteization of antibodies of clonal origin directed against catalase T. It demonstrates that such antibodies are suitable for immunoadsorption of labelled proteins from crude extracts.

EXPERIMENTAL PROCEDURES

The parental mouse myeloma cell line P3x63Ag8 was maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10 % fetal calf serum and 10 % donor horse serum (GIBCO), 10^{-4} M 8-azaguanine, penicilline (100 units/ml) and streptomycin (50 units/ml).

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Outbred albino mice were injected intraperitoneally with 100 µg of yeast catalase (see below) in 0.45 M NaCl/0.005 M Tris-HCl pH 7.0 emulsified in an equal volume of complete Freunds adjuvant and after five weeks received 100 µg catalase T into the tail vein. Eight days later, spleens were removed, dissociated mechanically and the cells washed once with phosphate buffered saline. Myeloma cells were washed once in serum-free MEM, mixed with the spleen cells (approximately 2.10^7 myeloma cells plus 2.10^8 spleen cells) and centrifuged.
Cell fusion was carried out in polyethyleneglycol-dimethylsulfoxide according to (3).
The cells were then seeded in HAT selection medium in 1 ml-aliquots (24-well plates) and incubated at 37° in a humidified atmosphere containing 5% CO_2. From two weeks after fusion, aminopterin was omitted from the medium.

Cell cloning was accomplished by limiting dilution of the cultures (1 cell/ml) in 24-well plates containing 1 ml/well and 2.10^5 mouse spleen cells/well as feeder cells.

Catalase T and catalase A were purified from baker's yeast and rabbit antisera specific for the two proteins were obtained as described previously. Immunoelectrophoresis was kindly carried out by Dr.H.Rumpold (Institut für Allgemeine und Experimentelle Pathologie der Universität Wien) as described (7).

The haploid Saccharomyces cerevisiae strain D 273-10B (ATCC 24657) was used to obtain radioactively labelled or unlabelled cell extracts. Cells were labelled with $^{35}SO_4^{2-}$ (50 µCi/ml, carrier-free, Amersham, U.K.) during growth in semi-synthetic medium containing 0.3 % glucose as carbon source. The medium described by Mason et al.(8) was modified by replacing sulfates by chlorides and adding yeast extract to a concentration of 1 g/l.

Direct immunoprecipitation was carried out as described previously (9). Immunoadsorption to Protein A-containing Staphylococcus aureus (Cowan I) cells were carried out by a modification of the method of Kessler (10). Crude extracts of ^{35}S-labelled yeast cells were obtained by breaking cells in homogenization medium (0.025 M Veronal-HCl, pH 8.2, 1% Triton X-100, 0.15 M NaCl, 0.01 % TPCK, TLCK and PMSF and a 1:100 dilution of Aprotinin solution) with a Braun homogenizer. The homogenate was centrifuged for 5 min in an Eppendorf Centrifuge and the supernatant solution was filtered through a 0.2 µm membrane filter.
Crude extracts were diluted 1:1 with homogenization medium containing 0.5 % deoxycholate and 0.5 M NaCl. Bacteria were washed 3 times before use with homogenization medium containing 0.5 % deoxycholate and 0.5 M NaCl. Antibodies were added to the extract; appropiate amounts were determined by titration curves. After 15 min at 4° C washed Staphylococcus aureus cells were added. After another 15 min incubation at 4° C the immunoadsorbent was collected by a 1 min centrifugation in an Eppendorf centrifuge and washed 3 times with homogenization medium containing 0.5 % deoxycholate and 0.5 M NaCl. Adsorbed immune complexes were solubilized by a 4 min treatment at 100° C with 2 % sodium dodecyl sulfate, 0.62 M Tris-HCl (pH 6.8), 10 % glycerol, 0.15 % dithiothreitol and protease inhibitors as described for the homogenization medium.

Dodecyl sulfate polyacrylamide gel electrophoresis was carried out on 10 % slab gels as described (11). Fluorography was performed according to Bonner and Laskey (12) but dimethyl sulfoxide was replaced by acetic acid. In some cases, bands

visualized by fluorography were cut out from gels and radioactivity was determined as described previously (13).

Enzymatic activity of catalase T was assayed as described (6). Activity units are μmoles H_2O_2 decomposed/min at 25° C. Proteolytic fingerprints of labelled proteins purified by immunoadsorption and dodecyl sulfate electrophoresis were obtained with Staphylococcus aureus V 8 protease (Miles Biochemicals) as described (14).

If not indicated otherwise, chemicals and biochemicals used were from Sigma, St.Louis.

RESULTS

Approximately 60 % and 40 %, respectively, of the cultures derived from two independent fusion experiments showed hybrid growth. To detect hybrids producing antibodies specific for catalase T, supernatants of the 48 hybrid culture obtained were pooled in 7 groups. These were tested for the presence of catalase T antibodies by immunoadsorption from ^{35}S-labelled yeast extracts containing catalase T to Protein A-containing Staphylococcus aureus cells. Only one of the pools turned out to be active in mediating adsorption of a protein with the electrophoretic mobility of catalase T (Fig. 1, lane 2). No such band was formed with supernatant from the parental P3x63Ag8 myeloma cells (not shown).

The supernatants combined in the active pool were then tested separately. Two of them, 21D3 and 21D4, were active (Fig. 1, lanes 4 and 5). Both cultures were cloned by limiting dilution; in both cases, 100 % of the clones showed activity, suggesting a clonal origin of the original cultures. Since subclones of 21D4 displayed considerably higher activity than subclones of 21D3, a subclone of 21D4 was used for all further experiments described here.

Denaturation of the labelled yeast lysate, followed by renaturation after dilution of dodecyl sulfate (15) and immunoadsorption with 21D4 antibodies gave a weakly positive reaction (Fig. 1, lane 6), whereas rabbit antisera efficiently recognise

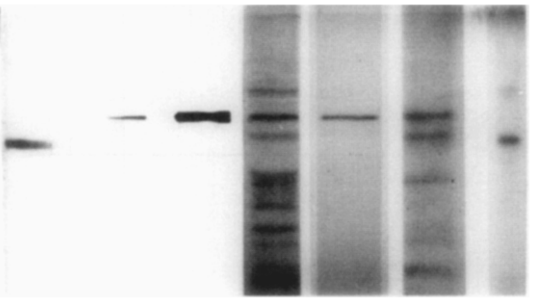

Figure 1: Characterization of specificity of hybridoma antibodies by dodecyl sulfate gel electrophoresis. Yeast catalases were immunoprecipitated from crude extracts of ^{35}S-labelled cells. Immunoprecipitated products were separated on 10 % dodecyl sulfate polyacrylamide gels and visualized by fluorography. Immunoadsorption with: lane 1: catalase A-specific antiserum; 2: pool 7 of hybridoma supernatants; 3: catalase T-specific rabbit antiserum; 4: supernatant from cell-line 21D3; 5: supernatant from cell-line 21D4; 6: 21D4 supernatant after denaturation of catalase T by boiling with dodecyl sulfate and 12 h renaturation after dilution of detergent (15). 7: Direct immunoprecipitation with 21D4 supernatant.

catalase T treated in this manner (16). Since the prosthetic group of catalase T, heme, is effectively removed from the protein by this method, this result shows that heme is not required for formation of the immune complex and that the antibodies obtained can recognize catalase T apoprotein. In contrast to immunoadsorption, direct immunoprecipitation with 21D4 antibodies gave no specific product (Fig. 1, lane 7), although catalase T can be quantitatively immunoprecipitated by this method with rabbit antisera (9).

Two further experiments gave final proof that 21D4 antibodies are directed against catalase T. As is illustrated in Fig. 2, enzymatic activity of catalase T could be quantitatively removed from extracts of yeast cells by immunoadsorption with 21D4 antibodies. The ^{35}S-labelled protein adsorbed with the help of the monoclonal antibody was subjected to limited proteolysis after purification on dodecyl sulfate gels (14) and was compared with authentic catalase T immunoprecipitated with specific

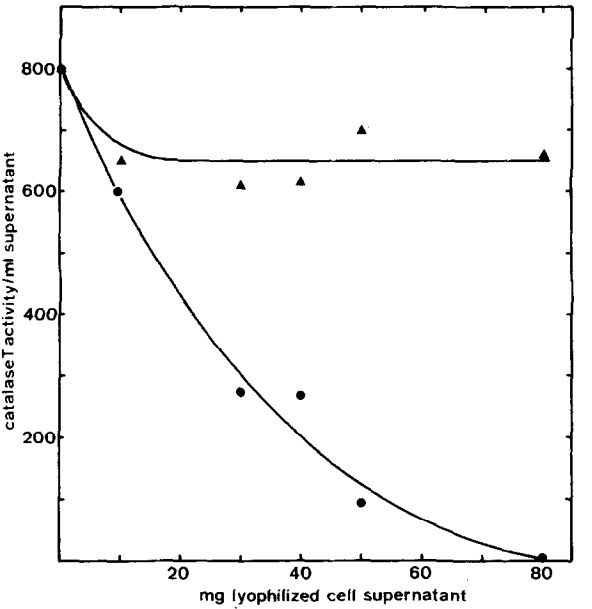

Figure 2: Titration of catalase T with monoclonal antibody. An unlabelled crude extract of yeast cells, grown to stationary phase, was pre-treated with catalase A-specific rabbit antiserum sufficient to precipitate all catalase A (6). After centrifugation of catalase A immunoprecipitate aliquots of supernatants were incubated with different amounts of 21D4 antibodies (●). Controls were carried out with supernatant from cell-line 21A6 producing no catalase antibody (▲). After immunoadsorption with Staphylococcus aureus cells catalase activity was assayed in the supernatant.

rabbit antiserum. Proteolytic fingerprints obtained for the two immunoprecipitates were identical (not shown).

Catalase T antibodies were produced by 21D4 cells when these were kept on medium containing 20 %, 2 % or 0 % serum. Yields of catalase T antibodies were quite similar in all three cases; approximately 2.6 µg catalase T could be immunoadsorbed with antibody present in 1 ml culture supernatant of 21D4 cells (cells were seeded at 10^6 cells/ml in fresh medium and incubated for 72 hours).

The initial clone of 21D4 was maintained in continuous culture for 8 months; during this time, no loss in antibody production was observed. Immunoelectrophoresis using specific

antisera showed that antibodies secreted by 21D4 cells belong to the IgG-type of immunoglobulins (not shown).

DISCUSSION

The results presented shown that antibodies suitable for immunoadsorption of labelled antigens from complex mixtures can be obtained from hybridoma cell lines. Antibodies produced by 21D4 hybrid cells recognize the enzymatically active tetrameric form of catalase T with great efficiency, but, in contrast to rabbit antisera, appear to react only poorly with heme-less catalase T protein. Further studies will have to show whether this difference in reactivity can be used in a differential assay of catalase T and its biosynthetic precursors (9, 17) or whether other monoclonal antibodies have to be isolated and characterized for this purpose. Although the antibody described here is highly active in immunoadsorption experiments, it appears to be inactive in direct immunoprecipitation.

As has been demonstrated in our study, monoclonal antibodies can be obtained in good yield from cells kept in the absence of serum. This observation should be of great importance for all those cases where antibodies free of other proteins, especially of other immunoglobulins, are needed, e.g. in the isolation of specific polyribosomes by immunoadsorption.

Although antibodies directed against individual antigens can be purified by affinity methods from mixtures of immunoglobulins, their isolation from supernatants of cultured hybridoma cells is certainly much more convenient. Large amounts of catalase T-specific antibodies can be obtained now without further need for purification of antigen. As purification of yeast catalases, like that of many other proteins, is

fairly tedious and timeconsuming, the availability of monoclonal cell lines producing such antibodies is of considerable practical value for further studies.

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