

18

Production of Monoclonal Catalytic Antibodies: Principles and Practice

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18.1

Introduction

Antibody catalysis is an experimental science. Although success in this field is dependent on conceptual insight and creative design, success reflects the quality of the experimental procedures. There are many conceptual steps on the way toward the realization of a new antibody catalyst, including mechanistic understanding of the specific reaction to be catalyzed, scholarly prediction of the transition state of highest energy, design of a chemically stable transition state analog, and planning of synthetic schemes for hapten, substrates, products, etc. Yet, the critical components of the entire effort are the experimental procedures, such as organic synthesis, immunization, screening, production of monoclonal antibodies, kinetic experiments, crystallographic studies, etc. Accordingly, we found it appropriate to devote one chapter in this book to the experimental work that is needed for the general production and purification of catalytic monoclonal antibodies via the hybridoma technology. No attempt is made here to cover any aspect of organic synthesis and kinetic studies which are specific to the chosen reaction to be catalyzed. We have focused on the experimental procedures that follow conjugation of the appropriate hapten to the carrier protein.

Although hybridoma technology is a mature and well-established method, it involves a variety of independent variables and steps. Each step can be carried out in many different ways, and the diversity of the published approaches reflects specific biological problems and experimental tradition that characterize any given laboratory. The literature offers a broad variety of methods, which differ from one another in speed, convenience, reproducibility, and cost. There are no right or wrong approaches, because every laboratory chooses the published strategies and adapts them to their specific needs. In this chapter we describe the methods of producing catalytic antibodies that have been used continuously over the past two decades in our laboratories at The Scripps Research Institute, without claiming that these methods are superior to others. For example, while the experimental literature offers many different immunization procedures with various antigen doses, time frames, and adjuvants, this account covers the procedures that have been working for us success-

fully over the years. These procedures are based on a number of leading references dealing with the production of monoclonal antibodies [1–3] and their purification [4, 5], as well as on the accumulating experience. Expectedly, the significant advance of chemical immunology within the past 15 years is reflected by the major improvements of protocols since an earlier description of these procedures was published in 1989 [6].

The hybridoma library obtained from immunization against a given hapten comprises a diverse population of binding proteins. An efficient method of screening this library is one of the keys to success in the field of antibody catalysis. The screening issue is not less important than either the understanding of the mechanistic details of the given reaction to be catalyzed or the rational design of hapten. The critical importance of good screening methods is highlighted by J.-L. Reymond in Chapter 10 of this book. Unlike strategies of rational design, including *de novo* synthesis of biocatalysts, as well as the preparation of organic and organometallic catalysts, the catalytic antibodies approach is characterized by a significant element of unpredictability. There are many facets of biocatalysis that are not yet fully understood, particularly those related to protein dynamics along the reaction coordinate. Even when some of this is understood, we do not yet know how to design the antibody active site accordingly. Therefore, the use of a transition state analog, even an optimal one, which is often impossible to make, represents only a “snapshot” of a continuous, dynamic process and is only a general guideline for the immune system. The resultant broadly diverse population of antibodies reflects the variety of ways in which the immune system can respond to this transition state analog. The beauty of this approach is the element of unpredictability, which is reminiscent of browsing scientific journals – a method by which one can find valuable items that were not looked for. In contrast, other methods, such as the imprinted polymers methodology, at their best provide the experimenter with what he or she has been searching for. Transition state binding is a parameter that can be well designed by immunization with an appropriate hapten. However, since there are several other parameters that lead to biocatalysis, the best hapten binder in a given antibody library may not necessarily be the best catalyst. Thus, although screening for binding is convenient and well established by traditional immunological methods, one should remember that in many ways it seems like searching for a lost coin under the street lamp. A much more efficient approach would be the screening for actual catalysis. Several methods have been used for such screening, including catElisa and the use of chromogenic and fluorogenic assays, all of which are covered by the specific chapter [7]. The screening methods described below represent those more generally used for hapten binding. These are the basics upon which improvements and screening for catalysis may be designed.

18.2

Immunization

Not all hapten conjugates will generate a strong antibody response even if the exact immunization protocol is followed. Variables that can influence the *anti*-hapten

response include the length of the spacer group or linker, the hapten density on the carrier protein, the choice of carrier protein, the stability of the conjugate, the strain of mice, and the adjuvants used. We generally use 8–12 week old mice for immunization. These 129GIX+ mice have routinely given us the best response to small organic hapten and peptide conjugates. However, we have also successfully used Balb/c, Swiss Webster, A/J, and C57 mice. We use RIBI's Adjuvant System (RAS) MPL+TDM (# R700, Corixa), which gives as good a response as Complete Freund's Adjuvant (CFA) and sometimes better, and has none of the adverse side effects associated with CFA. We have also been very successful using ALUM (#77161, Pierce) for boosts.

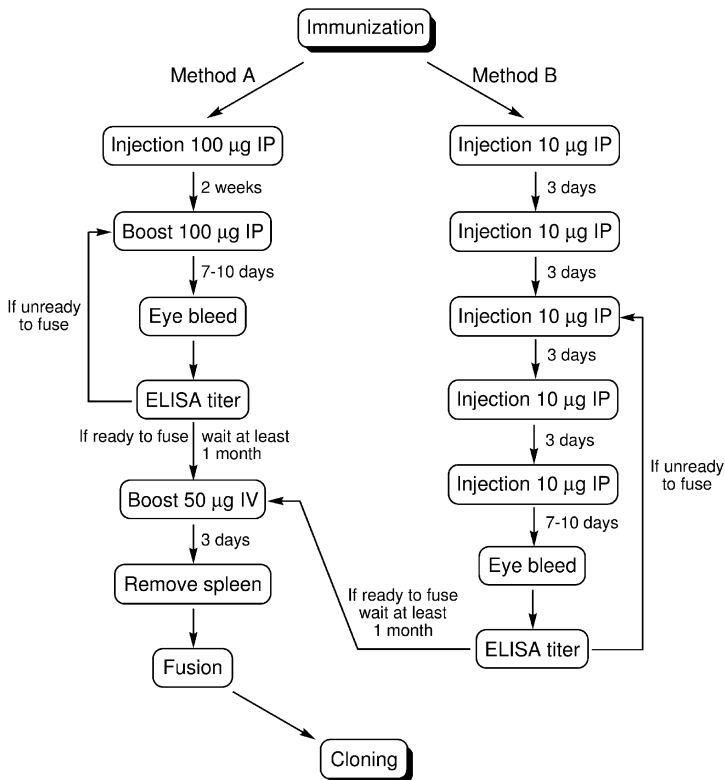


Fig. 18.1

In general, we immunize between 4 and 8 mice per hapten using two different immunization strategies in an attempt to get a stronger response to weaker immunogens. The first method is the one we have used for many years (Fig. 18.1). Each mouse receives a solution of 100 µg hapten-KLH conjugate in 100 µL phosphate-buffered saline (PBS: 10 mM sodium phosphate, 160 mM sodium chloride, pH 7.4) mixed with an equal volume of RAS reconstituted in PBS. The mice are injected intraperitoneally (IP) using a 23 gauge needle. After two weeks, they are given a second injection exactly similar to the first (100 µg per mouse in RAS, IP). Approximately 7–10 days later, the mice are bled and the serum titer determined by ELISA. If the

titer (dilution giving 50% of the maximum absorbance) is less than 6400, the mice are given additional boosts of 50–100 µg after 2–3 weeks until the titer is 12 800 to 25 600 or more. Once a sufficient titer is reached, we wait at least 1 month for the circulating antibody levels to decrease before giving the mouse a final injection of 50 µg KLH conjugate in 100 µL PBS intravenously (IV) in the lateral tail vein. The spleen is removed 3 days later for fusion.

The second method, which we have been using additionally throughout the last year, uses lower antigen amounts, with more frequent injections. 10 µg hapten conjugate in 50 µL PBS per mouse mixed with an equal volume of RAS is injected IP, and this is repeated every 3 days (4 more times) over a 2 week period. About 7–10 days later, the mice are bled, and the serum titer is determined by ELISA. If it is found that additional boosts are required, the mice are boosted with three 10 µg boosts, 3 days apart, in RAS. As with the higher-dose immunization, once a sufficient titer is reached, we wait at least 1 month for the circulating antibody levels to decrease before giving each mouse a final injection of 50 µg KLH conjugate in 100 µL PBS intravenously (IV) in the lateral tail vein. The spleen is removed 3 days later for fusion.

18.3

Hybridoma Production and Screening

At least one week prior to the fusion, we thaw a fresh vial of myeloma cells. It is critical that the myeloma cells be healthy and in log phase on the day of the fusion in order for it to be successful. Cells that have been growing too long and have come out of log phase or cells that have not been growing long enough to achieve log growth will not fuse optimally. We try to keep the cell density between 3 and 6×10^5 /mL. It is important to choose a myeloma line that has been selected to be a non-producer of IgG. We use the X63-Ag8.653 line because it is a non-producer and has high fusion efficiency.

The spleen cells from the hyper-immunized mouse and myeloma cells are washed 3 times with 30 mL RPMI 1640 media (supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 50 µg/mL gentamycin) and mixed together in a 5:1 ratio (spleen:myeloma) in a 50 mL conical tube. After the final spin, it is important to aspirate the media completely, then to spread the pellet out by gently tapping the tube. It is also important that the cells be spread along the bottom edge of the conical so that all the cells have equal access to the PEG. Addition of 1 mL 50% PEG 1500 (BMB #783-641) that is pre-warmed to 37 °C is done drop-wise over 1 min using a 1 mL syringe and an 18 g needle, while gently rotating and tapping the tube to re-suspend the cells. The PEG is then slowly diluted out with 1 mL RPMI-1640 media over 1 min, and then 8 mL over 2 min. The cell membranes are very fragile at this point. The cells are then placed in a 37 °C water bath for 10 min and centrifuged. The supernatant is decanted and the cells are gently re-suspended in 5 mL complete media (supplemented RPMI-1640 with 10% FCS). The cells are again placed in a 37 °C water bath for 10 min and are then added to 225 mL HAT media (RPMI-1640

supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 µg/mL gentamycin, 10% FCS, 0.1 mM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine). They are plated into fifteen 96-well plates (Corning 3596, 150 µL/well). The fusion is fed 50 µL/well with HT media (RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 µg/mL gentamycin, 10% FCS, 0.1 mM hypoxanthine, and 16 µM thymidine) on days 4, 8, and 12. By this time, macroscopic colonies should be seen. We generally get growth in 40–60 % of the wells, so we assay for antigen binding directly from the 96 well plates.

Having a sensitive enzymatic assay that could detect catalytic activity at the antibody concentrations in cell supernatant (1–20 µg/mL) would greatly facilitate the screening process and eliminate the labor-intensive procedures of subcloning, producing, and purifying large numbers of antigen binders that are non-catalytic. If it is possible to determine background rates and sensitivity levels of the assay ahead of time so that screening is done directly from the 96-well plate, this greatly increases the chances of finding catalysts. We initially screen 600–900 hybridomas for binding, but only a small subset of these is carried through subcloning and large-scale antibody production and purification to be tested for catalytic activity. As the hybridomas are expanded and moved from the 96-well plate to the 48-well, 24-well, etc., we continually monitor their binding by ELISA to the hapten coupled to BSA. We characterize the hybridomas by titer and isotype, keeping only IgG subclasses that have stable titers of 16 or above. We also rank them by their affinity for related ligands and substrates using a competitive inhibition assay.

Our ELISA procedure can be modified or adapted if necessary, depending upon the nature of the hapten being used. Since we regularly immunize with a KLH-conjugate, it is important to have another carrier protein for screening purposes. BSA is easy to conjugate and does not aggregate and precipitate easily, so that the plate is evenly coated. The hapten-BSA conjugate is diluted to 50 µg/mL in PBS and plated at 25 µL/well into a 96-well 1/2-area EIA plate (Corning 3696) and allowed to dry overnight at 37 °C. The antigen is fixed to the plate with 50 µL/well methanol for 5 min. The methanol is then shaken out and the plates allowed to air dry for 5–10 min. Non-specific binding sites are blocked with 50 µL/well of “BLOTTO” (5% w/v non-fat powdered milk in PBS) for 30 min at room temperature. The excess BLOTTO is shaken out, and the primary antibody is immediately added. The plates should not be allowed to dry out from this point on, because this may lead to non-specific binding. Addition of 25 µL/well of the primary antibody diluted in BLOTTO, (1:1 for tissue culture supernatant, 1:100 for mouse sera, and 1:1000 for ascites) is followed by incubation in a moist chamber for 1–2 h at 37 °C. The plates are washed 10 times with de-ionized water using a showerhead. It is recommended to alternate the direction of wash in order not to miss any well. The excess water is shaken out and 25 µL/well of the secondary antibody are added, followed by goat *anti*-mouse-horseradish peroxidase conjugate (Southern Biotech) diluted 1:2000 in BLOTTO, and the plates are then incubated for 1 h at 37 °C in a moist chamber. When isotyping the hybridomas, we use the HRP clonotyping kit (Southern Biotech) with conjugates specific for each of the murine heavy and light chains. The plates are washed 10 times with de-ionized water and 50 µL/well of the developer (30 mL 0.1 M citrate buffer, pH

4.0, with 9 μL 30% H_2O_2 , 200 μL 45 mg/mL ABTS) is added. The plates are then read in an ELISA reader (Molecular Devices V-max kinetic plate reader) at 414 nm after 30 min.

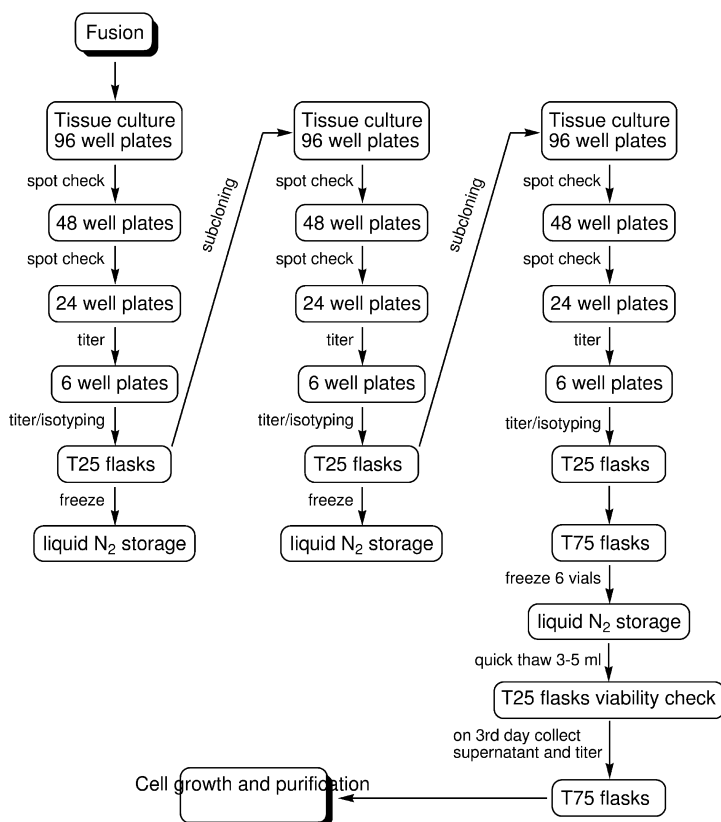


Fig. 18.2

The hybridomas are subcloned through at least 2 generations to guarantee monoclonality (Fig. 18.2). We subclone by limiting dilution sometimes 3 or 4 times if the hybridoma does not appear to be stable. Newly established hybridomas that have not been growing long in culture do not grow well when plated out at limiting dilutions, but it is important to start subcloning the hybridomas as soon as possible to avoid the cells being taken over by non-producers. We take both of these issues into consideration by titering the individual wells repeatedly while they are being expanded, so that we can tell when they are beginning to lose activity. In this way we can wait to subclone until the cells are dividing well in culture. We generally have a large number of hybridomas to subclone, so we clone by limiting dilution without counting the cells. We make a series of dilutions that result in plating out approximately 0.5 and 5 cells per well in 200 μL in a 96 well plate (48 wells at each density). It takes 10–14 days for macroscopic colonies to show up. We pick 8–16 different clones per hybridoma to move up into a 48 well (depending upon the initial titer of the hybridoma), trying

to take wells that appear to have only one clone. At this point many of the clones will not be positive by ELISA. We pick the 6 best to move up to a 24-well plate, and at this point we titer all the subclones. We recheck the titer and isotype at the 6-well stage and pick the best one to freeze down and subclone again. The whole process is repeated a second and third time until all clones from a 96-well plate show positive binding, with identical isotypes and equal titers. At this point we feel relatively secure that we have a monoclonal stable line, and we do a large-scale multiple freeze-down of the hybridoma with 6 identical aliquots of cells.

In order to have a frozen stock of cells with greater than 95% viability it is important to have the cells dividing well and in log phase. We freeze the cells when we have 50 mL of 4 to 6×10^5 cells/mL. The cells are pelleted, re-suspended in 3 mL of freezing media (90% fetal calf serum and 10% DMSO), and aliquoted 0.5 mL per cryovial. The vials are placed immediately into a cryo-container (Nalge 5100-0001) at -80°C , which allows for a controlled, slow rate of freezing (1°C per min). After 4–6 hours the cells are then placed in a liquid nitrogen storage tank. After 2–3 days we thaw one of the vials to make sure it is viable and not contaminated. The vials are thawed quickly in a 37°C water bath and the cells placed in 10 mL of complete RPMI-10% FCS. The cells are then pelleted to remove any traces of the DMSO and then re-suspended in 5 mL media. We culture the cells for at least 48 h to make sure they are viable and start dividing, and then we test the supernatant for antibody titer. Once we have the cloned hybridoma cells backed up in liquid nitrogen, we can start large-scale antibody production.

18.4

Large-Scale Antibody Production

There are a number of different techniques that can be used to produce the monoclonal antibodies, depending upon the amount of antibody required, the facilities available, the time frame, and the available budget. In the past we have grown our hybridomas exclusively in mice for ascites production. We have made an effort in recent years to find alternatives to ascites production in order to reduce painful procedures using animals. At this time we only use ascites when we have tried a few different methods and have been unsuccessful in producing our antibody *in vitro*.

If we need 30 mg or less of purified antibody we produce it in T-flasks (Fig. 18.3). Hybridoma cells will typically produce 2–50 $\mu\text{g}/\text{mL}$ of antibody, depending upon the isotype of the clone and the cell density. Antibody levels can be maximized by growing up to 500–1000 mL of cells and allowing them to grow until they start dying (sometimes 10–14 days). The supernatant is then collected, centrifuged, filtered through a $0.2\ \mu\text{m}$ filter, concentrated by ultra-filtration, and purified. We have been able to obtain as much as 60 mg this way, but 30–40 mg is the average. On traditionally low producers like IgG3 antibodies, the yield does not exceed 20 mg.

For larger amounts of antibody we have been very successful in growing the hybridomas in Integra Bioscience's CELLline flasks. Once established, we routinely get 15–20 mg/harvest from each CL-1000 flask, and the flasks can be grown for 2–3

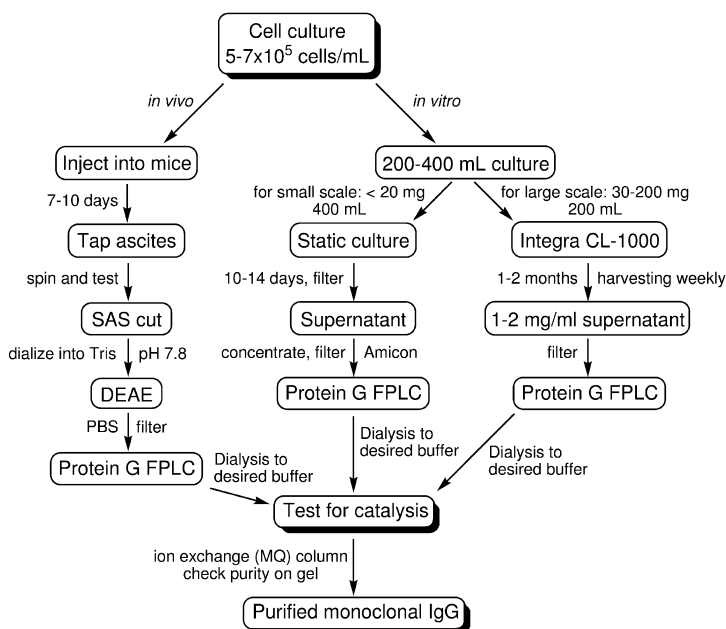


Fig. 18.3

months and harvested once or twice per week, resulting in 150–300 mg of antibody. When we need 1–2 g antibody, we simply inoculate 5–6 CL-1000s. The initial cost of the *in vitro* antibody production in the CELLLine flasks higher than in antibody production in ascites, but, barring any contamination, the flasks can be grown for months with continual harvests, bringing the overall cost per mg of antibody down. Purification of the antibody is easier as well.

Before starting any large-scale antibody production it is critical that the hybridomas are well cloned with a stable titer and are in the log phase. When inoculating a CL-1000 flask, we have had the best results using 200 mL of 5 to 7×10^5 cells/mL, ($\sim 1 \times 10^8$ cells). The cells are centrifuged at 1200 rpm and re-suspended in 15 mL of complete RPMI supplemented with 20% FCS, 50 I.U./mL penicillin, and 50 μ g/mL streptomycin. One liter of complete RPMI, with “pen-strep” (but without FCS) is placed in the basal chamber of the flask. Then the 15 mL of cells are pipetted into the cell compartment, removing any trapped air bubbles. After one week we check the viability of the cells with trypan blue. If the viability is less than 50%, we generally grow up more cells to re-inoculate. If viability is 50% or greater, we will do a partial harvest of the cells (the exact amount will vary with the total number of cells). We try to put $\sim 5 \times 10^7$ viable cells in 15 mL of 20% FCS back into the cell compartment. The rest of the harvested cells are spun, and the supernatant is collected and filtered through a 0.2 μ M filter and purified. We change the basal media (one liter of complete RPMI with pen-strep without FCS) every week and harvest the cell compartment. After approximately 4 weeks we can usually harvest the cell compartment twice per week, resulting in close to 30–40 mg of antibody per week for good producers.

Occasionally we find a cell line that will not adapt to growing at high densities in the CL-1000. If we need to produce over 50 mg we will use ascites production. Since we generally immunize 129GIX+ mice and the myeloma cell line we use (X63 Ag8.653) was isolated from Balb/c mice, we need make our ascites in a hybrid strain, Balb/c X 129GIX+. The mice are primed with 0.2 mL of pristane (2,6,10,14-tetramethyl pentadecane, Aldrich) intraperitoneally at least one week prior to injecting the cells. An amount of 50 mL of 5×10^6 cells/mL are spun, washed once in PBS, and re-suspended into 2 mL of PBS. We then inject 500 mL of cells per mouse intraperitoneally into 4 mice. After 7–14 days the ascites should begin to form. The mice can be “tapped” sometimes as many as 3 times in a 7 day period, resulting in 10–20 mL of ascites per mouse. The ascites fluid is centrifuged at 3000 RPM for 10 min to remove the blood cells and stored at -20°C until it is purified. We get between 0.5 and 15 mg of antibody per mL of ascites depending on the hybridoma. We have found that most IgG1s produce 10–15 mg/mL, IgG2as produce 5–7 mg/mL, IgG2bs produce 2–4 mg/mL, and IgG3s produce 0.5–2 mg/mL.

18.5

Antibody Purification

For the purification of antibodies made in T-flasks or CL-1000s, we affinity purify the supernatant over Protein G (Fig. 18.3). We use Gammabind™ plus Sepharose™ (Amersham Pharmacia Biotech AB, 17-0886-04) and Hi Trap Protein G HP, 5 mL pre-packed columns (Amersham Pharmacia Biotech AB, 17-0405-01), depending upon the amount of antibody being purified and the machine (FPLC or AKTA Prime) being used. We bind the antibody in PBS, pH 7.4, and wash with 3 column volumes to make sure all unbound proteins are removed. We then elute with 0.1 M acetic acid (pH 3.0) and neutralize the eluted antibody with 1M TRIS, pH 9.0. The antibody is then dialyzed into PBS pH 7.4, concentrated by ultra-filtration, sterile filtered, and stored at 4°C . For long-term storage, we aliquot the antibody and freeze it at -20°C .

For purification of ascites, we start by precipitating the antibody using saturated ammonium sulfate (SAS). We slowly add the ice-cold SAS dropwise to the ascites while stirring on ice and let it sit on ice for at least 15–20 min, then centrifuge at 9000–10000 rpm for 15 min. Doing a 50% cut gives a slightly higher antibody yield, but it can precipitate additional serum proteins as well. A 45% cut is generally cleaner, but more antibodies are left behind. The pellet is re-suspended in PBS and extensively dialyzed to remove the excess salt. We switch the dialysis buffer from PBS to 50 mM TRIS, pH 7.8 (or 8.0 for IgG2as), and for our second purification step we use an ion-exchange column. We use DEAE-Sephacel™ (Amersham Pharmacia Biotech AB, 17-0500-01), which has a strong positive charge at pH 7.8 and 8.0. Since immunoglobulins are the most basic of all serum proteins (with isoelectric points between 6 and 8), at pH 7.8 the IgG will be eluted first. We elute using a step gradient of increasing NaCl concentration, (50 mM, 75 mM, 100 mM, 150 mM, 250 mM, and 500 mM). We test all fractions by ELISA and concentrate the antibody-containing

ones by ultra-filtration. The antibody is then dialyzed into PBS to be affinity purified on Protein G (as above).

18.6

Testing for Catalytic Activity

Once we have made a panel of purified antibodies, we dialyze them into the appropriate buffer for each catalytic reaction. Most antibodies will tolerate a pH of between 5 and 9 without too much trouble. If it is necessary to use a buffer without any salt, it helps to keep the antibody in solution if the molarity of the buffer is increased to 100 mM or more. For pH extremes or low-ionic-strength buffers, we dialyze a very small amount of antibody first using a slide-a-lyzer (Pierce, 66415). This lets us know if our antibodies will tolerate the chosen buffer system. If we see any precipitation of the antibody, we have the opportunity to modify the buffer without losing our entire antibody.

Once we have identified potential catalysts, we purify the antibody through additional columns to rule out any enzyme contamination. We will use Source S and Source Q anionic and cationic exchange columns (Amersham Pharmacia Biotech AB) with a linear salt elution, and gel-filtration columns using HiPrep 26/60 Sephacryl S-200 High Resolution (Amersham Pharmacia Biotech AB, 17-1195-01). The purified antibody is analyzed by SDS-PAGE to check for contaminating proteins. If the antibody retains its catalytic activity throughout all of the purification tests and can be inhibited, we grow it up a second time, going through all of the purification steps, to make sure it is reproducible.

18.7

Preparation of Fab, F(ab')₂, and Fab' Fragments

Antibody fragments, mainly Fab (50 000 dalton), are prepared primarily for structure determination by X-ray crystallography. In some cases they may be prepared for catalysis when either a monovalent catalyst is required or in order to verify that catalysis occurred exclusively in the binding site.

Antibodies may be conveniently considered as having three protein domains, two identical antigen binders, Fab, and an effector domain, Fc. Antibodies can be fragmented by partial digestion with either papain or pepsin (Fig. 18.4) [4]. Papain treatment produces two Fab fragments and one Fc fragment. In contrast, pepsin treatment can be used to release two antigen binding domains still bound together, F(ab')₂. The different mouse subclasses vary in their susceptibility to enzyme digestion to form antibody fragments. Mouse IgG2a and IgG2b are easily digested with papain to form two Fab fragments and one Fc fragment (Fig. 18.4). As mouse IgG1s are more resistant to papain, we generally use pepsin to form the F(ab')₂, which can then be reduced by cysteine to form the Fab' fragment.

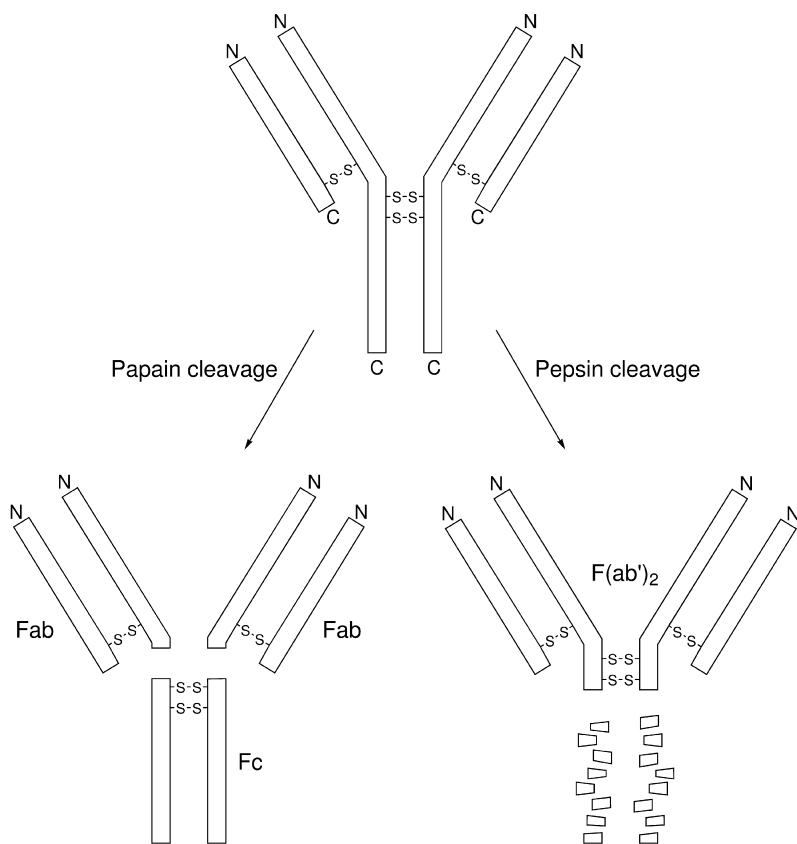


Fig. 18.4

Before starting any large-scale digestion, it is important to run small test digests, varying the cysteine concentration, enzyme concentration, and digestion time, to determine the optimal digestion conditions using all fresh reagents. The sample is dialyzed into sodium acetate (0.1 M, pH 5.5). When using mercuripapain (Sigma P-9886), the enzyme needs to be pre-activated for 30 min at 37 °C in Buffer A (100 mM sodium acetate, pH 5.5, 1 mM EDTA, 50 mM cysteine). For IgG2b we have found that 40 µg of papain per mg of antibody is a good starting enzyme concentration. For IgG2a we have been able to get complete digestion with as low as 20 µg of papain per mg of antibody.

When there is a good supply of antibody it saves time to set up 3 or 4 different tubes with different amounts of enzyme (20, 30, 40, and 50 µg) using 5 mg of antibody per test digest in 1 mL total volume. EDTA (1/100 volume of 100 mM) and 50 µl of pre-activated papain solution are then added. The tubes are sealed, mixed well, and placed in a 37 °C water bath. Aliquots (20 µl each) are taken at 0.5, 1, 2, 4, 6, and 24 h to determine the optimal digestion time. Iodoacetamide (2 µl, 0.75 M, 1/10 volume) is then added to inactivate the papain, and the mixture is incubated for 30 min at room

temperature. Samples are stored at 4 °C until the digestion mixture is analyzed using SDS-PAGE. If the digestion of the IgG is still incomplete, the test digest is repeated with increased concentration of cysteine. When the optimal conditions are achieved, the digest reaction is scaled up.

To purify the Fc and Fab, the sample is first dialyzed into PBS and then purified on a Protein A column. The Fc (and any remaining IgG) are retained on the column while the Fab and enzyme are eluted. The Fc can then be eluted with 0.1 M acetic acid, pH 3.0. The crude Fab can then be loaded onto a protein G column and eluted with 0.1 M acetic acid, pH 3.0. All fractions are analyzed by SDS-PAGE. If the Fab is still not sufficiently pure, it is further purified on an IEF gel to determine its pI. Dialysis into the appropriate pH buffer is carried out before purification on either an anionic or cationic exchange column. In most cases we use 20 mM PB, pH 7.0, to bind the protein on a cationic exchange column (Source S) and elute it with a linear gradient from 0 to 500 mM NaCl in 20 mM PB, pH 7.0. Finally, the sample is dialyzed into PBS, pH 7.4.

For IgG1 antibodies, the sample is dialyzed into 0.1 M sodium acetate, pH 7.0, and then into 0.1 M sodium acetate, pH 3.8, immediately before digesting. Fresh pepsin (Sigma, P-6887) stock solution is prepared at 10 mg/mL in 0.1 M sodium acetate, pH 3.8. The test digestion is carried out at 37 °C with 5 mg/mL antibody, various pepsin concentrations (1–4% by weight), and various incubation times (0.5, 1, 2, 4, 6, and 24 h). Samples of 20 µL each are taken at each time point, the reaction is stopped by adding 1/10 volume of 2 M TRIS pH 8.0, and the degree of digestion is analyzed by SDS-PAGE. Too long a digestion time may result in decreased yields. If the digestion of IgG is incomplete, lowering of the pH may facilitate it. Once the digestion conditions are optimized, the digestion can be scaled up. The digestion mixture is dialyzed into PBS, pH 7.4, and loaded on a Protein A column to bind any undigested IgG. Immunopure A Binding Buffer (Pierce, 21001) will increase IgG1 binding to Protein A. This works with Immunopure Mouse IgG1 Mild Elution Buffer (Pierce, 21022). Once the IgG is removed, the mixture is loaded onto a Protein G column in PBS and eluted with 0.1 M acetic acid to remove the enzyme. At this point the mixture should contain only F(ab')₂ with a single band of 100 000 dalton on SDS-PAGE. If further purification is required, the pI of the sample should be determined, and it is then purified on an ion-exchange column. Most of our F(ab')₂s have had a pI of 7.5–8.0, and we have run a Source S cationic exchange column in 20 mM PB, pH 6.5. To generate the Fab', the sample needs to be reduced with cysteine. For that purpose, 100 mM cysteine solution is prepared in dH₂O. A set of experiments is carried out with the antibody sample (3 mg/mL) using multiple tubes with different cysteine concentrations (2.5–10 mM) and various times (1–4 h). The reaction is stopped by the addition of iodoacetamide (20 µl, 0.75 M) per 1 mL sample and the mixtures are analyzed by SDS-PAGE. After achieving satisfactory conditions, the reaction is scaled up and the resultant Fab' is then dialyzed into PBS, pH 7.4.

18.8**Conclusion**

We are continually exploring new ways of making and identifying catalytic antibodies. These include, for example, improving our immunization procedures to elicit tighter binders with higher affinities for the substrates, testing out new media formulations and growth conditions to improve subcloning efficiencies, and developing more sensitive catalytic assays that can be used to detect catalytic activity from cell supernatant instead of purified antibody. All of these would shorten the time between hapten design and synthesis and identification of antibody catalysts.

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