Fragmentation of Immunoglobulin G

To study the distinct regions of an immunoglobulin, it is useful to cleave them with proteolytic enzymes into various fragments (see Fig. 16.4.1 for a schematic of the structure of antibody molecules and their proteolytic fragments). The Fc portion of the antibody molecule is responsible for certain biological effects of antibodies, such as binding to Fc receptors on cells, mediating antibody-dependent cellular cytotoxicity, and complement fixation. It is also thought to be the more immunogenic portion of the molecule in vivo. For experiments concerning effects mediated by specific binding of antibody to cells in vitro or localization of antibodies to certain organs in vivo, it may be necessary to eliminate the nonspecific effects of Fc-receptor binding. The affinity of antibody for an antigen on the surface of viable cells can only be measured accurately using monovalent antigen-binding fragments (Fab).

Classically, fragmentation to the monovalent Fab fragment is carried out using papain digestion (see Basic Protocols 1 and 2), and the bivalent F(ab’)_2 fragment is obtained by pepsin digestion (see Basic Protocols 3 and 4). Although papain digestion produces Fab fragments from IgG of all subclasses from all species, pepsin is not so universally useful. Alternative methods of fragmentation to F(ab’)_2 include use of papain that is preactivated with cysteine (see Alternate Protocol 1) and use of the enzyme ficin (see Alternate Protocol 2). These alternate methods are particularly useful for mouse IgG1 antibodies. When using pepsin and papain for the first time on an antibody, pilot experiments are performed to determine the correct reaction conditions; with preactivated papain and ficin these pilot experiments are unnecessary.

![Figure 16.4.1](image_url)

Figure 16.4.1 Proteolytic digestion fragments of immunoglobulins. Structural domains of the variable and constant regions of the light chain (VL, CL) and heavy chain (VH, CH1, CH2, CH3), as well as interchain disulfide bonds (S-S), are indicated. Monovalent fragments of IgG produced by papain digestion are known as Fab, while bivalent papain fragments are F(ab)2. F(ab’)2 refers to the bivalent fragments of IgG produced by pepsin digestion; those derived from IgM are called F(ab’)2µ. Bivalent subunits of IgM with both Fab and Fc are known as IgMs (subunit), and fragments of IgG containing a single antibody-binding site and an intact Fc portion are Fab/c.
PILOT FRAGMENTATION OF IgG TO Fab USING PAPAIN

Papain digestion of IgG produces Fab fragments. This protocol is designed to determine conditions for large-scale fragmentation, and can be used for IgG from mouse (all subclasses), rat, human, goat, sheep, horse, chicken, guinea pig, and cow. Once the method for a particular antibody is established, it will be reproducible for each fragmentation.

Purified monoclonal antibody is digested with papain in the presence of the reducing agent cysteine. To determine the optimal conditions, both the concentration of papain and the time of digestion are varied. After terminating the digestions, the fragments are dialyzed and analyzed by SDS-PAGE. By assessing the gel results, optimal conditions are chosen. These conditions are used in the large-scale fragmentation of IgG to Fab (see Basic Protocol 2).

Materials

- 2 mg/ml purified IgG (UNIT 16.3) in PBS (UNIT 16.3)
- Papain (2× crystallized suspension; Sigma)
- Digestion buffer (see recipe)
- 0.3 M iodoacetamide (prepare fresh from crystalline material; Sigma) in PBS
- PBS (UNIT 16.3)
- Additional reagents and equipment for nonreducing gel electrophoresis (UNIT 6.5) and dialysis of proteins (APPENDIX 3C)

1. Pipet 100 µl of 2 mg/ml purified IgG in PBS into each of 24 numbered microcentrifuge tubes.

2. Prepare two papain solutions in 5 ml digestion buffer—one at 0.1 mg/ml papain and the other at 0.02 mg/ml.

   Because papain is a suspension, mix well without vortexing before pipetting.

3. Add 100 µl of 0.1 mg/ml papain to eight of the IgG tubes. Add 100 µl of 0.02 mg/ml papain to a second group of eight IgG tubes. Finally, add 100 µl digestion buffer without papain to the remaining eight tubes. Close the lids on all of the tubes.

   Thus, reactions have been set up with enzyme/antibody (E/A) ratios of 1:20 and 1:100, and control tubes with no enzyme.

4. Prepare a digestion time curve by incubating tubes in a 37°C water bath and removing them at different times in groups of three—one tube each of E/A at 1:20 and 1:100, and one control tube. Remove the first group after 1 hr, and the subsequent groups at 2, 4, 6, 12, 18, 24, and 48 hr. As each group of three tubes is removed from the water bath, terminate the reaction by adding 20 µl of 0.3 M iodoacetamide in PBS and then vortexing.

   The final concentration of iodoacetamide (0.03 M) irreversibly inactivates papain.

5. Dialyze the reaction mixture (APPENDIX 3C).

   Dialysis can be performed using a homemade microdialysis chamber or commercial microdialysis cassettes (e.g., Slide-A-Lyzer Dialysis Cassettes, Pierce).

6. Analyze digestion products by loading an 80-µl aliquot of the dialyzed mixture from each tube onto a 10% nonreducing polyacrylamide gel (UNIT 6.5).

   Antibodies that have been digested with papain in the presence of cysteine to produce Fab fragments show a major protein band on a nonreducing polyacrylamide gel at a molecular weight of ~50 kDa, and a second band at 27 kDa which is the remaining Fc portion. There are usually a couple of smaller bands at 24 and 15 kDa which are other unimportant products of digestion. Obviously, undigested antibody will appear as a band at 150 kDa. Frequently, effects of cysteine alone are seen in antibody which has not been treated with papain, causing a protein band on the gel at 120 kDa. This 120-kDa band is also seen in...
papain, causing a protein band on the gel at 120 kDa. This 120-kDa band is also seen in the digestion mix at short incubation times (up to 4 hr). The digestion is complete when no protein bands of 150 kDa or 120 kDa are seen in the gel lanes containing the digestion mixture. The time of complete digestion will vary between antibodies.

The effect of incubation with cysteine alone will be seen in the control lanes, probably because cysteine causes some unfolding of the antibody molecule, thus altering its electrophoretic mobility. Fab fragments have a molecular weight of 50 kDa (see Table 16.3.2). At early time points some intact antibody is expected on the gel, with the reaction moving towards completion as incubation times increase.

The amounts of antibody used in this method are suitable for analysis by SDS-PAGE using standard 150 × 1.5–mm gels. If a minigel apparatus is available (e.g., the Phast system from Pharmacia), modify the amounts accordingly and use less antibody.

7. Choose the optimal conditions to obtain Fab from the IgG by assessing the gel results and use these conditions for Basic Protocol 2.

LARGE-SCALE FRAGMENTATION OF IgG TO Fab USING PAPAIN

Exact conditions for optimum yield of Fab from each antibody should be assessed from the pilot experiment (see Basic Protocol 1); those conditions are then scaled up in this procedure. The procedure is similar to Basic Protocol 1 where papain is used to digest the antibody and the digested antibody is dialyzed. The dialyzed antibody fragment is then purified by protein A–Sepharose affinity chromatography (UNIT 16.3) and can be further purified by size-exclusion chromatography (APPENDIX 3). The purity of the product is assessed by nonreducing gel electrophoresis (UNIT 6.5).

Materials

- Papain (2× recrystallized suspension; Sigma)
- Digestion buffer (see recipe)
- ≥1 mg/ml IgG (≥5 mg total; UNIT 16.3) in PBS
- Iodoacetamide crystals
- PBS, pH 8.0 (UNIT 16.3)
- Protein A–Sepharose CL-4B
- Sephacryl S-200 Superfine (Pharmacia Biotech)
- 5 × 100–mm column (Bio-Rad)
- 26 × 900–mm column (Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (APPENDIX 3C), column chromatography (UNIT 16.3 & APPENDIX 3), concentrating protein solutions (APPENDIX 3C), and nonreducing gel electrophoresis (UNIT 6.5)

1. Dissolve the required amount of papain in a volume of digestion buffer equal to the volume of antibody solution to be digested; use optimum amounts of enzyme and antibody as well as incubation times from the results of the pilot fragmentation in Basic Protocol 1.

   Digestion buffer is made at twice the required final strength so that when added to the antibody solution in equal volume, the mixture will be at the correct molarity with respect to EDTA and cysteine. Because papain is a suspension, mix well without vortexing before pipetting.

2. Add the papain buffer solution to the ≥1 mg/ml IgG in PBS, mix, and incubate for the required time (determined from Basic Protocol 1) in a 37°C water bath.

3. Stop the reaction by adding crystalline iodoacetamide to 0.03 M final. Mix carefully to ensure that the iodoacetamide dissolves completely.
4. Transfer to dialysis tubing and dialyze the mixture against 2 liters PBS, pH 8.0, for 12 to 20 hr at 4°C.

More detailed procedures for large-volume dialysis, and selection and preparation of dialysis membranes can be found in APPENDIX 3C.

5. Prepare a 5 × 100-mm protein A–Sepharose CL-4B column (UNIT 16.3 & APPENDIX 3) and load dialyzed mixture onto it. Collect unbound fraction containing the Fab fragment and enzyme. If necessary, wash column with PBS to completely recover Fab fragments.

The remaining intact antibody will bind to the column.

6. Concentrate the mixture containing the Fab fragments to ≤5 ml (APPENDIX 3C).

7. Prepare a 26 × 900-mm Sephacryl S-200 Superfine size-exclusion column (APPENDIX 3) and load concentrated mixture onto it. Collect fractions corresponding to a molecular weight of 50 kDa.

Fractions can be collected and their molecular weight assessed by SDS-PAGE or a precalibrated size-exclusion column.

8. Check the purity of the final product (1 to 80 µl) on a 10% nonreducing polyacrylamide gel (UNIT 6.5).

9. Assess the concentration of the Fab fragments at $A_{280}$ (Table 16.3.2). Store fragments in borate buffer (UNIT 16.3) at 4°C or in PBS containing 0.02% NaN₃ at −70°C.

**BASIC PROTOCOL 3**

PILOT FRAGMENTATION OF IgG TO F(ab′)₂ USING PEPSSIN

Pepsin fragmentation of IgG to produce F(ab′)₂ is similar to the pilot fragmentation with papain (see Basic Protocol 1) except that the pH and time of digestion are varied. The optimum pH for pepsin digestion is ~2; however, this low pH may damage the antibody, so a pH of ~4 is the minimum recommended. Two different pH values are tested in this pilot experiment, as some antibodies will fragment to Fab or smaller fragments at lower pH values. Because pepsin treatment can be slightly harsher than papain, an enzyme/antibody (E/A) ratio of no higher than 1:20 is recommended, as it is possible that pepsin could damage the binding site of the antibody molecule.

*NOTE:* F(ab′)₂ fragments cannot be obtained from IgG₂β. Treatment of IgG₂β with pepsin will result in fragments of about the correct molecular weight (120 kDa), but these will be Fab/c fragments (consisting of one Fab fragment and the Fc portion; Fig. 16.4.1).

**Materials**

3 mg/ml purified IgG (UNIT 16.3)

Acetate buffer, pH 4.0 and 4.5 (see recipe)

0.1 mg/ml pepsin (Sigma) in pH 4.0 and pH 4.5 acetate buffers

2 M Tris base

PBS (UNIT 16.3)

Additional reagents and equipment for protein dialysis (APPENDIX 3C) and nonreducing gel electrophoresis (UNIT 6.5)

1. Dialyze 2 ml of 3 mg/ml purified IgG against 200 ml acetate buffer, pH 4.0, and another 2 ml of 3 mg/ml purified IgG against 200 ml acetate buffer, pH 4.5, both for 4 hr at 4°C.

2. Determine the concentrations of both dialyzed IgG solutions by $A_{280}$ readings using the acetate buffer of corresponding pH as the blank (Table 16.3.2).
3. Adjust each dialyzed antibody solution to a concentration of 2 mg/ml in the acetate buffer of corresponding pH.

4. Pipet 100 µl of the 2 mg/ml IgG, pH 4.0, into each of 16 numbered microcentrifuge tubes. Into another 16 numbered microcentrifuge tubes, pipet 100 µl of the 2 mg/ml IgG, pH 4.5.

   Thus, there are two groups of 16 tubes—one group at pH 4.0 and the other at pH 4.5.

5. Add 100 µl of 0.1 mg/ml pepsin in acetate buffer, pH 4.0, to eight tubes in the IgG, pH 4.0 group. To the other eight tubes in this group, add 100 µl of acetate buffer, pH 4.0. Add 100 µl of 0.1 mg/ml pepsin in acetate buffer, pH 4.5, to eight tubes in the IgG, pH 4.5 group. To the other eight tubes in this group, add 100 µl of acetate buffer, pH 4.5.

   Thus, there are two groups of 16 tubes with pepsin at an E/A ratio of 1:20 at two different pH values, and control tubes without enzyme.

6. Incubate the 32 tubes in a 37°C water bath, removing one tube from each group of eight tubes after 1, 2, 4, 6, 12, 24, and 48 hr. As each tube is removed, stop the reaction by adding 40 µl of 2 M Tris base.

7. Transfer to dialysis tubing and prepare microdialysis chamber (APPENDIX 3C). Dialyze the samples against 1 liter PBS for 4 hr at 4°C.

8. Analyze 80-µl aliquots of the fragmentation products on a 10% nonreducing polyacrylamide gel (UNIT 6.5).

   F(ab′)_2 has a molecular weight of 110 kDa. Bands of this molecular weight should be seen in the fractions treated with pepsin. Lower-molecular-weight bands (~50 kDa) are probably the Fab′ fragment of the antibody, a further degradation product from pepsin. Amounts of F(ab′)_2 and Fab′ produced will vary among antibodies, depending on the fragmentation conditions and individual susceptibilities.

9. Choose the best conditions to obtain maximum yield of F(ab′)_2 by assessing the gel results and use these conditions in the following basic protocol.

**LARGE-SCALE FRAGMENTATION OF IgG TO F(ab′)_2 USING PEPсин**

As with the papain large-scale protocol, optimal fragmentation conditions should be assessed for each antibody from the pilot fragmentation (Basic Protocol 3). Purification is by protein A–Sepharose affinity chromatography (Basic Protocol 2). It is important to remember that IgG_2b generally cannot be fragmented to F(ab′)_2.

**Materials**

- ≥1 mg/ml purified IgG (UNIT 16.3)
- Acetate buffer at appropriate pH (see Basic Protocol 3 and recipe)
- 0.1 mg/ml pepsin in acetate buffer at appropriate pH (see Basic Protocol 3)
- 2 M Tris base
- PBS, pH 8.0 (UNIT 16.3)
- Protein A–Sepharose CL-4B
- Sephacryl S-200 Superfine (Pharmacia Biotech)
- 5 × 100–mm column (Bio-Rad)
- 26 × 900–mm column (Pharmacia Biotech)
- Additional reagents and equipment for protein dialysis (APPENDIX 3C), concentrating protein solutions (APPENDIX 3C), column chromatography (UNIT 16.3 & APPENDIX 3), and reducing and nonreducing gel electrophoresis (UNIT 6.5)

**BASIC PROTOCOL 4**
Digest IgG
1. Dialyze ≥1 mg/ml IgG against acetate buffer at the appropriate pH.
2. Determine the concentration at A_{280}.
3. Add 0.1 mg/ml pepsin dissolved in acetate buffer at appropriate pH to give an E/A ratio of 1:20 (see Basic Protocol 3, step 5). Incubate the reaction mixture for the required time (see Basic Protocol 3, step 6) in a 37°C water bath.
4. Stop the reaction by adding ~50 µl of 2 M Tris base/ml reaction. Check with pH paper that pH = 8.0 and add more Tris base if necessary.
5. Transfer the mixture to dialysis tubing and dialyze against 1 liter PBS, pH 8.0, at 4°C (APPENDIX 3C).

Purify F(ab')₂
6. Prepare a 5 × 100–mm protein A–Sepharose CL-4B column (UNIT 16.3 & APPENDIX 3) and load dialyzed mixture onto it. Collect the unbound fraction.

   At this stage, the F(ab')₂ fragment can be reduced to Fab’ if desired. Add cysteine to a concentration of 0.01 M, mix well and incubate 2 hr at 37°C. Cysteine should reduce and alkylate the inter–heavy chain disulfide bonds. If further alkylation is thought necessary, make the mixture 0.15 M with respect to iodoacetamide. Purify the Fab’ using the steps below.

7. Concentrate the mixture to ≤5 ml.
8. Prepare a 26 × 900–mm Sephacryl S-200 Superfine size-exclusion column (APPENDIX 3) and load concentrated mixture onto it. Collect fractions corresponding to a molecular weight of 110 kDa.

   Measure the protein eluting from the column with a UV monitor or spectrophotometer. Assess their contents by SDS-PAGE or use of a precalibrated column.

9. Check the purity of the final product (1 to 20 µl) on a 10% SDS–polyacrylamide gel under reducing and nonreducing conditions (UNIT 6.5).

   F(ab’)₂ will give a single band at 110 kDa on a nonreducing gel and a single band or a doublet at 25 kDa on a reducing gel.

10. Assess the concentration of the F(ab’)₂ fragments at A_{280} (Table 16.3.2). Store fragments in borate buffer (UNIT 16.3) at 4°C or in PBS containing 0.02% NaN₃ at −70°C.

**ALTERNATE PROTOCOL 1**

**FRAGMENTATION OF IgG USING PREACTIVATED PAPAIN**

Mouse IgG₁ has shown significant resistance to pepsin digestion. The antibody is missing a leucine at residue 234; this restricts the hinge region and causes pepsin resistance. This method can be used to obtain F(ab)₂ fragments from IgG₁. Additionally, Fab fragments can be obtained from mouse IgG₂a and IgG₂b by this cleavage. It is a gentler procedure and can give a more stable fragmentation and a high yield of fragments. The procedure is time-consuming but incubation periods are usually less critical. The conditions used here are typical of large-scale fragmentation and purification. It is not necessary to carry out a pilot fragmentation experiment to assess conditions when using this procedure, although the type of fragment obtained varies between subclasses.
**Prepare papain**

1. Dialyze 10 mg IgG in 2 to 5 ml PBS in acetate/EDTA buffer (*APPENDIX 3C*).
2. Determine the concentration at $A_{280}$ with the acetate/EDTA buffer as a blank.
3. Incubate 2 mg/ml papain and 0.05 M cysteine for 30 min in a 37°C water bath.
   - *Papain requires a free sulfhydryl group for its catalytic activity. In the native crystalline form, this group is blocked and the enzyme exhibits extremely low proteolytic activity. Cysteine activates the group and activation is optimal in the presence of a heavy-metal binding agent such as EDTA.*
4. Equilibrate a PD-10 column with 20 ml of acetate/EDTA buffer (*APPENDIX 3*).
5. Apply papain/cysteine (from step 3) to the PD-10 column. Collect ten 1-ml fractions, eluting with acetate/EDTA buffer.
   - *This process removes the cysteine from preactivated papain. Use preactivated papain within 2 hr of preparation and keep on ice until use.*
6. Assay the fractions at $A_{280}$ and pool the two or three fractions containing papain. Calculate the preactivated papain concentration using the following formula:
   $$A_{280}/2.5 = \text{mg preactivated papain/ml}.$$  

**Digest IgG**

7. Add 0.5 mg preactivated papain to dialyzed, 10-mg IgG solution (from step 2) and vortex. Incubate 6 to 12 hr in a 37°C water bath.
8. Stop reaction by adding crystalline iodoacetamide to a final concentration of 0.03 M.
9. Dialyze against 1 liter PBS, pH 8.0, at 4°C for 6 to 12 hr (*APPENDIX 3*).

**Purify fragments**

10. Prepare a protein A–Sepharose column equilibrated in PBS, pH 8.0 (*UNIT 16.3*), and apply reaction mixture. Collect unbound 2-ml protein fractions and pool those represented by the first peak.
   - *The antibody fragments will elute in the first protein peak. Assess this using a UV monitor or spectrophotometer.*
11. Concentrate the unbound fractions to ≤5 ml (*APPENDIX 3C*).
12. Prepare a size-exclusion (SE) column (*APPENDIX 3*) and load the sample. Collect 100 fractions (1% of the column volume). Assess the fractions containing the appropriate molecular weight by SDS-PAGE (*UNIT 6.1*) or on a precalibrated column. Store in borate buffer (*UNIT 16.3*) at 4°C or in PBS containing 0.02% NaN$_3$ at −70°C.
   - *Monitor the fractions with a UV spectrophotometer at $A_{280}$ nm. The fragments obtained from this procedure are Fab from IgG$_{2a}$ and IgG$_{2b}$ and F(ab)$_2$ from IgG.$_1$*
FRAGMENTATION OF MOUSE IgG₁ TO F(ab′)₂ USING FICIN

IgG₁ can also be fragmented to F(ab′)₂ by treatment with ficin in the presence of cysteine. Ficin is a thiol protease isolated from fig latex.

**Additional Materials** *(also see Basic Protocol 4)*

- 10 mg mouse monoclonal IgG₁ *(UNIT 16.3)* in 2 to 5 ml PBS
- 50 mM Tris/2 mM EDTA, pH 7
- Ficin solution: 1 mg/ml ficin (Sigma) in 50 mM Tris/2 mM EDTA, pH 7
- Cysteine
- 100 mM N-ethylmaleimide (Sigma)
- PBS *(UNIT 16.3)*

Additional reagents and equipment for protein dialysis *(APPENDIX 3C)* and column chromatography *(UNIT 16.3 & APPENDIX 3)*

1. Dialyze 10 mg mouse monoclonal IgG₁ in PBS against 1 liter of 50 mM Tris/2 mM EDTA, pH 7, for 6 hr at 4°C.
2. Determine the concentration of the antibody by reading the A₂₈₀ with the dialysis buffer as a blank.
3. Add ficin solution to give a 1:30 enzyme/immunoglobulin ratio.
4. Add solid cysteine to the mixture to give a final cysteine concentration of 1 mM.
   
   *Cysteine is required to activate the enzyme.*
5. Incubate 4 to 5 hr in a 37°C water bath with gentle shaking.
   
   *The incubation time is not critical.*
6. Stop reaction by adding 1/10 vol of 100 mM N-ethylmaleimide.
7. Dialyze the mixture against PBS, then purify the fragments by protein A–Sepharose and size-exclusion (SE) chromatography *(see Alternate Protocol 1, steps 10 to 12 and UNIT 16.3 & APPENDIX 3).*

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

**Acetate buffer, pH 4.0 and 4.5**

Bring 0.2 M sodium acetate to pH 4.0 and 4.5 (as required) with glacial acetic acid.

**Acetate/EDTA buffer, pH 5.5**

Bring 0.1 M sodium acetate to pH 5.5 with glacial acetic acid and add EDTA to 0.003 M.

**Digestion buffer**

*PBS (UNIT 16.3) containing:*

- 0.02 M EDTA (disodium salt)
- 0.02 M cysteine

Prepare fresh; store <10 hr on ice
Background Information

Immunoglobulins can be cleaved by proteolytic enzymes into functionally distinct subunits (Fig. 16.4.1). Generally, fragments that bind antigens are of interest. Fab and F(ab’)_2 fragments contain the antibody-binding portion of the molecule; the Fc fragment is generally only of interest if studying the crystal structure of antibodies. The Fab or F(ab’)_2 portion of the antibody may be required in certain experiments. For instance, in mixed lymphocyte response (MLR) and cytolytic T lymphocyte (CTL) assays, the Fc portion may cause artifacts because it binds to cells bearing the Fc receptor. It has also been suggested that the Fc portion of the antibody may be more immunogenic in vivo (Hellstrom and Hellstrom, 1985). The Fc portion of IgG2a and IgG3 can invoke antibody-dependent cell-mediated cytotoxicity responses in vivo and in vitro.

The monoclonal antibodies routinely used for in vitro and in vivo experiments are from the mouse, and the protocols of this unit are designed for use with mouse antibodies. Fragmentation of antibodies from other species can be evaluated and optimized using the pilot protocol provided. Fragmentation with papain is similar for many species.

Subclass-dependent susceptibility of mouse antibodies to proteolytic cleavage has been widely reported (Parham, 1986). The order of sensitivity to proteolytic enzymes has been found to be IgG2a > IgG1 > IgG2b > IgG1. Not all antibodies, however, fall into this ranking; IgG2b can be extremely sensitive to the action of papain in the presence of cysteine. If such sensitivities are found, then the method in which cysteine is removed from the enzyme following preactivation is recommended (see Alternate Protocol 1; Parham et al., 1982).

It must be stressed that all monoclonal antibodies are different and, for this reason, standard conditions for fragmentation are not given for papain and pepsin digestion. A pilot experiment should always be performed to assess optimal conditions before carrying out a large-scale fragmentation. The pilot experiment is very similar to the large-scale procedure. Microdialysis (Overall, 1987) is recommended so that the conditions from small- to large-scale are identical.

IgG can be fragmented to yield monovalent Fab fragments using the proteolytic enzyme papain in the presence of the reducing agent cysteine. IgG subclasses (e.g., IgG1 and IgG2) have different susceptibilities to papain digestion. IgG1, IgG2a, and IgG3 can be digested to bivalent F(ab’)_2 fragments using pepsin. Although IgG1 may be sensitive to pepsin degradation, a more stable digestion is achieved using papain in the absence of cysteine. Ficin is also useful for digestion of IgG1 to F(ab’)_2 (Mariani et al., 1991). It is a thiol protease purified from fig latex. It requires activation with cysteine; the concentration of cysteine determines the extent of digestion. Generally, IgG2b cannot be fragmented to F(ab’)_2. Prior to fragmentation, a pilot experiment should be undertaken to determine individual conditions for fragmentation of each antibody. Separation of fragments can be carried out very efficiently by ion-exchange (IEX) chromatography on DE52 (UNIT 16.3 & APPENDIX 3); other methods for separation have been described in the protocols.

Pepsin cleavage of mouse IgG subclasses results in two sites of proteolytic sensitivity, one on either side of the inter–heavy chain disulfide bonds as suggested by Parham (1983). Primary cleavage may occur at a site on the COOH-terminal side of the bridges, giving F(ab’)_2, followed by secondary cleavage on the NH2-terminal side, giving Fab’.

Critical Parameters

Because each monoclonal IgG is different, if one antibody of a particular subclass is cleaved under a certain set of conditions, it cannot be expected that all other antibodies of that subclass will behave in the same manner. A pilot experiment should always be carried out when a new antibody is to be fragmented. It is probably better to choose a reaction time that will leave some antibody unfragmented rather than risk damaging the antibody-binding site by excessive exposure to proteolytic en-
zymes. Intact antibody can be easily removed from the fragmentation mixture by passage through a protein A–Sepharose column. For those antibodies that do not bind well to protein A, protein G–Sepharose (UNIT 16.3) or ion-exchange chromatography (APPENDIX 3) are reasonable alternatives.

Troubleshooting
Optimum reaction conditions may vary after scaling up from the pilot fragmentation. Occasionally the incubation times appear shorter in the small-scale procedure, so it may be necessary to increase the incubation time a little when scaling up.

Another possible difficulty is that the fragmentation does not proceed at all. Probably, this is related to the enzyme. Ascertain that there are no enzyme inhibitors, such as azide, present in the antibody solution (UNIT 16.3) before ordering new enzyme.

Anticipated Results
Yields are frequently subclass-dependent. A 100% yield of Fab fragment would be twice the number of moles of intact antibody in the starting mixture, and a 100% yield of F(ab')2 would be equal to the number of moles of antibody in the starting mixture. Typically, yields of 45% to 55% are achieved, although some investigators report yields as high as 70% to 80%.

Time Considerations
Dialysis is usually carried out overnight, although 4 hr is sufficient for samples up to 100 ml. Running a protein A–Sepharose column takes ~4 hr, and running a size-exclusion column takes overnight.

Dialysis and running of columns are time-consuming procedures. The total time required to obtain fragments from a new antibody—beginning with the pilot experiment to the end of the large-scale production—is 4 to 5 days for Fab fragments and 6 to 8 days for F(ab')2 fragments.

Literature Cited


Key Reference
Parham, 1983. See above.

A lucid and comprehensive guide to the production of fragments from mouse monoclonal antibodies.

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