17 chapter

Immunoassays

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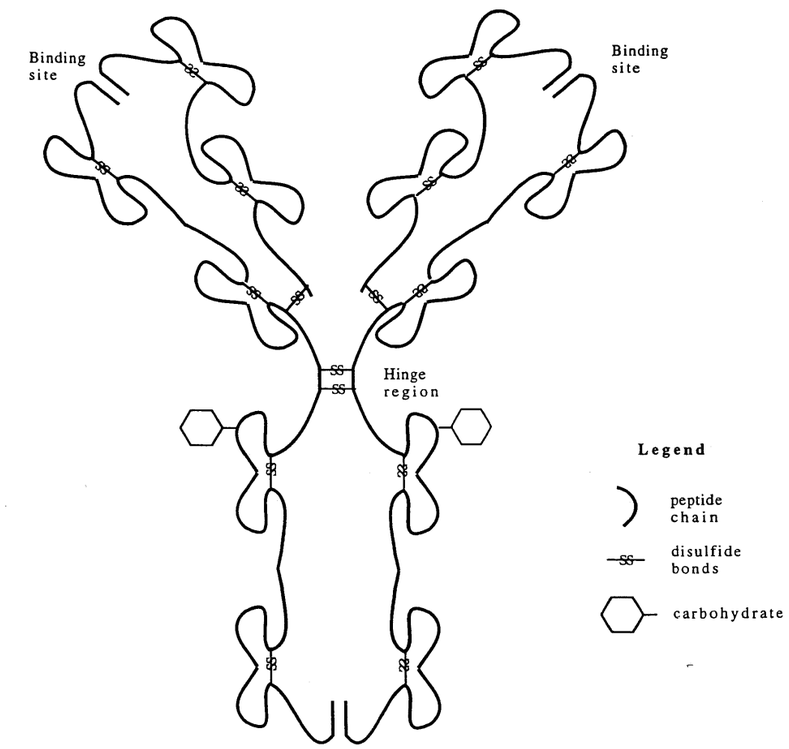
17.1 INTRODUCTION

Immunology is a relatively new science that has developed rapidly in the last few decades. Immunoassays are one of the most useful analytical developments associated with this new science. While immunoassay variations are too numerous to cover completely in this chapter, there are several procedures that have become standard for food analysis because of their specificity, sensitivity, and simplicity. Immunoassays are widely used for food residue analysis, identification of bacteria and viruses, and protein detection. Protein detection is important for determination of allergens, meat species authentication, and detection of genetically modified plants. With any new field a vocabulary develops for important terms. To fully understand immunoassays some of these terms need to be defined.

The two key parts of any immunoassay are antigens and antibodies. An **antigen** is any molecule that induces the formation of antibodies. **Antibodies** are proteins produced by animals in response to an antigen. These proteins bind the particular antigen responsible for their induction.

Antibodies can develop remarkably strong binding affinities for their antigens. These affinities are among the strongest noncovalent interactions known between molecules. It is not unusual to develop affinity constants for antibody to antigen binding that are 10^{12} L/mol. This means that at equilibrium, with similar concentrations of antibody and antigen, there would be a trillion antigens bound for every single free antigen!

Since the antibody and antigen are central to any immunoassay, it is useful to better understand the basic structure of the antibody and how it binds the antigen. Figure 17-1 is a stylized picture of an antibody. The antibody is a Y-shaped molecule made up of four polypeptide chains that are linked by inter- and intra-disulfide bonds. Two of the polypeptide chains are identical and roughly twice as large as the other two identical polypeptide chains. Because of their relative sizes, the former pair is known as **heavy chains** and



17-1 figure Antibody structure.

the latter pair as **light chains**. Overall, an antibody is a very large protein of approximately 150,000 molecular weight.

Antigen is bound by two identical binding sites made up of the end portions of a heavy and light chain at the top of the Y. Different antibodies produced by different B cells can have many variations in amino acid sequences near the binding sites for both the heavy and light chain. This leads to a tremendous diversity of binding sites for different antibodies. For example, a mouse has 10^7–10^8 different antibodies (and at least this number of different B cells), each with a unique binding site. The rest of the antibody (away from the binding site) is quite consistent, and small variations in this region result in different antibody classes. Figure 17-1 is actually an example of the most common class of antibody found in a mammal's serum, **immunoglobulin G**, or simply, IgG.

To understand immunoassays, the most important part of the antibody to study is the binding site. The antigen binding site is a cleft between loops of a heavy and light polypeptide chain. From a molecular point of view this cleft is quite large. Experiments with carbohydrates have indicated that the binding site is filled by a dextran oligosaccharide composed of seven glucose units. Put another way, the molecular weight of the portion of the antigen that can occupy the cleft is about 1000 g/mol.

Understanding the binding site of the antibody helps to further define the bound antigen. The antibody binds to the outside of the antigen in a specific region. This specific region bound by a single antibody binding site is known as an **epitope**. Moreover, the binding of the antibody to the antigen does not involve covalent bonding, but the same interactions that are responsible for the tertiary structure of proteins. These interactions include electrostatic, hydrogen bonds, and Van der Waals. While the latter interactions, Van der Waals, are the weakest, they often can be the most important because every atom can contribute to the antibody–antigen binding as long as the atoms are very close to each other (generally about 0.3–0.4 nm). This requirement for very close proximity is why antibody to antigen bonding is considered something like a lock and key interaction, where the surfaces of the antibody binding site and the antigen epitope are mirror complements of each other.

A major variable in an immunoassay is the type of antibody used. When serum antibody is used from any animal, there are many different antibodies that bind different epitopes on the antigen. This collection of different antibodies is known as **polyclonal** antibodies. Scientists knew that individual B cells produced antibodies with only one binding site, but were unable to culture B cells outside of the animal. However, in 1975, Köhler and Milstein (1) successfully fused cancer, or myeloma cells, with B cells. The new fused cells, or **hybridomas**, retained the properties of both of the parent cells. That is, they could be cultured, like cancer cells, and produced antibodies like the B cells. Antibodies produced with this procedure became known as **monoclonal** antibodies. Monoclonal antibodies are identical in every way and bind antigen with only one type of binding site; that is, a single epitope is bound. Moreover, the hybridomas were "immortalized" by the procedure and with proper care could produce as much identical antibody as required. It did not take the scientific community long to appreciate the tremendous advantages of these monoclonal antibodies and Köhler and Milstein were awarded the Nobel Prize for their work in 1984. While monoclonal antibodies are initially much more expensive to produce there is the possibility for limitless identical antibody, often from non-animal sources such as large-scale production of the hybridomas in cell growth chambers. These advantages outweighed the initial development costs for many immunoassay manufacturers.

17.2 THEORY

All immunoassays require two things. The first is that there must be some method to separate or differentiate free antigen from bound antigen. Secondly, antigen or antibody must be quantifiable at low concentrations for maximum sensitivity. Detection at very low concentrations has required very active labels. One of the first successful immunoassay procedures was developed by Yalow and Berson (2) in 1960. This procedure used radioactive iodine, I^{131}, a "hot" radioisotope with a half-life of only eight days. This rapid radioactive decay allowed for the second requirement of immunoassays, quantification at low concentrations. Yalow and Berson used paper chromato-electrophoresis to separate antibody-bound antigen from free antigen fulfilling the first requirement of an immunoassay. For other antigens, a variety of techniques for separation were developed, including adsorption of free antigen with charcoal or selective precipitation with ammonium sulfate or polyethylene glycol. With all the variations in separation, however, the radioactive iodine labeling remained and these assays became known as **radioimmunoassays** or RIA.

A common feature of proteins that is useful in immunoassays is the binding of proteins to various hydrophobic surfaces. Proteins have large regions that contain hydrophobic groups that prefer not to be exposed to water. These nonpolar hydrophobic groups include hydrocarbons and aromatic groups that prefer to interact with similar groups, rather than a polar solvent such as water. In aqueous conditions these regions will bind to other hydrophobic surfaces with

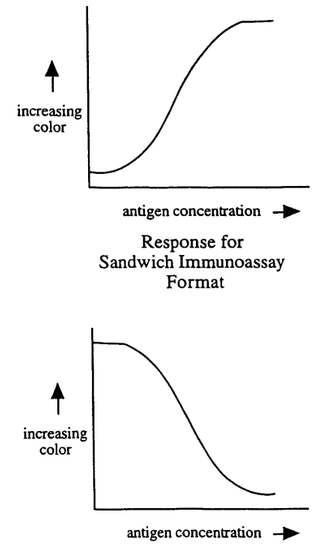
Van der Waals interactions, excluding water. Surfaces commonly introduced in immunoassays to take advantage of this type of binding include charcoal, nitrocellulose, and plastic. Plastic containers of many types are used very commonly for immunoassays. Among the most popular are microplates made of plastics such as polystyrene. These microplates typically are formatted to contain 96 individual wells, each with a capacity of about 300 µl of solvent. To differentiate the wells, the vertical rows are labeled A to H and the columns numbered 1 to 12. It is important to realize that proteins bind to the bottom and sides of the wells in these plates randomly through hydrophobic interactions. The hydrophobic interactions between proteins and these surfaces increase at lower temperatures (due to less molecular motion) and with increased ionic strength of the solvent (increasing solvent polarity). Also, detergents can coat hydrophobic surfaces and must be used with care so that they do not interfere with binding of proteins to a hydrophobic surface.

17.3 ENZYME IMMUNOASSAY (ELISA) VARIATIONS

17.3.1 Overview

While RIAs worked well, they were confined to specially equipped laboratories because of the dangers associated with the use of radioactive iodine. Immunoassays did not develop for more general use, including field use, until enzyme labels were developed. Pioneers in this development were Engvall and Perlmann (3) who in 1971 developed an immunoassay that they called an **enzyme-linked immunosorbent assay**, or ELISA. They also helped to popularize the use of separation of proteins using hydrophobic plastics such as polystyrene. These developments expanded the use of immunoassays to so many research areas that the term ELISA is still commonly used to describe all enzyme immunoassays.

The ideal enzyme for an ELISA or enzyme immunoassay is one that is very stable, easily linked to antibodies or antigens, and rapidly catalyzes a noticeable change with a simple substrate. In the final step of an ELISA, remaining enzyme reacts with a substrate, to generate a molecule that is colored and can be quantitated spectrophotometrically. The type of spectrophotometer used to monitor color development caused by the enzyme action is called an **ELISA plate reader**. Surprisingly, with the many enzymes available, one enzyme, **horseradish peroxidase**, is by far the most popular (4). Horseradish peroxidase is very stable, with many procedures now developed for attaching it covalently to a variety of molecules. Most important, however, is the fact that this enzyme has



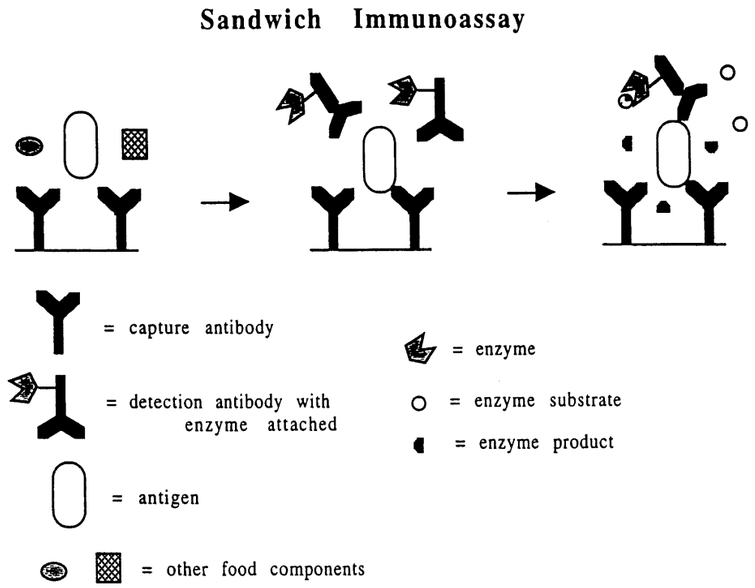
17-2 figure Relationship between color development and antigen concentration for different immunoassay formats.

a very fast catalytic rate with many colorless substrates to produce color. Other enzymes used for immunoassays include alkaline phosphatase, β-galactosidase, and urease. After horseradish peroxidase, alkaline phosphatase has been the next most popular enzyme for enzyme immunoassays.

Three enzyme immunoassay variations are described in the sections that follow: sandwich, competitive, and indirect. The indirect immunoassay variation can be applied to both sandwich and competitive formats. For example, one variation might be an indirect competitive immunoassay. With the sandwich ELISA format, the amount of color development is directly related to the amount of antigen present in the sample. With any competitive ELISA format, there is an inverse relationship between the amount of color developed and the amount of antigen present in the sample (Fig. 17-2).

17.3.2 Sandwich Immunoassays

One of the most popular formats for an enzyme immunoassay is the antibody **sandwich immunoassay** (Fig. 17-3). The "meat" in the antibody sandwich is the antigen. This immunoassay format is most commonly used for identification of proteins. In food analysis this



17-3 figure Sandwich immunoassay.

can involve identifying an adulterant, such as pig protein in a beef product; or a protein allergen such as peanut protein; or wheat protein in a product that would be a problem for people suffering from celiac disease.

Generally an antibody that binds to the antigen is first immobilized in some manner. The most common immobilization of the antibody is simply binding it to a hydrophobic surface such as plastic. Excess antibody is removed by washing and the test is ready for analysis of a food extract. The immobilized antibody is called a **capture antibody**. The food solution being tested contains many compounds that might act as antigens. However, the antibody was prepared by immunization of an animal with a specific, purified protein antigen, and only this protein antigen in the food solution will bind to the capture antibody. Now the antigen and the capture antibody are immobilized and the remaining food solution can be washed away. Note that the arrows between sections in Fig. 17-3 indicate a washing step followed by introduction of another solution. After the washing step, another antibody bound to an enzyme is introduced. This antibody, called the **detection antibody**, also recognizes the antigen. Again excess detection antibody is washed away, then colorless enzyme substrate is added to develop a color if bound enzyme is present. Enzyme will only be present if the detection antibody has been immobilized by binding to antigen. The greater the color development, the greater the amount of antigen present. That is, there is a direct proportionality between the amount of color seen in the final step and the amount of antigen present in the extracted food sample. To increase the sensitivity of a sandwich immunoassay, one can use more antibodies for capture of the antigen. This immunoassay format can be made very sensitive and remarkably specific since two antibodies must detect the antigen.

In the simplest version of the sandwich immunoassay, a polyclonal antibody solution is divided into two parts. One part is bound to plastic to become the capture antibody. The second portion of the polyclonal antibody solution is bound to an enzyme like horseradish peroxidase and becomes the detection antibody. Monoclonal antibodies also can be used, but now care must be exercised since a single type of monoclonal antibody cannot be used for both the capture and detection antibodies since only one unique epitope is recognized by any monoclonal antibody. Put another way, the antigen must be able to bind two antibodies at the same time and therefore must have at least two distinct epitopes recognized by different antibodies. However, if two different monoclonal antibodies recognizing two distinct antigen epitopes are used, the incubation with the food extract and the detection antibody actually can be carried out in one step.

17.3.3 Competitive Immunoassays

17.3.3.1 Problems Associated with Assay of Small Molecules

Because of selectivity, sensitivity, and the directly proportional relationship between color development and amount of antigen detected, the sandwich immunoassay is the format of choice for any large molecule. However, many of the molecules analyzed in food are not as large as proteins but are small molecules such as toxins, or antibiotic and pesticide residues. In these cases there are several problems that must be overcome before an immunoassay can be prepared.

The first problem is that when animals are injected with small molecules, they do not develop antibodies against these molecules. Generally a molecule must be greater than 5000 molecular weight to be perceived as an antigen by an animal's immune system. The solution is to covalently link the small molecule, or some appropriate derivative of the small molecule, to a large carrier molecule. The linked form of the small molecule is known as a **hapten**. The most common molecules used as carriers are proteins that are fairly soluble for simplicity in chemical linking, and foreign to the animal to properly stimulate an immune response. Typical carrier molecules include albumin proteins from a different species, such as bovine serum albumin and hemocyanins that are obtained from crustaceans. Of course when a hapten-protein conjugate is used for immunization of an animal, its immune system is stimulated to produce antibodies that bind not only the externally attached hapten and its covalent link but also the exposed exterior of the foreign protein.

A second problem in developing an immunoassay for a small molecule is that a sandwich immunoassay format will not work since two different epitopes are required for both antibodies to bind. A small molecule represents only one epitope or even only part of one epitope.

The solution to the problems described above is to use a **competitive immunoassay** format (Fig. 17-4). The first step required in a competitive immunoassay involves immobilizing the small molecule, often as a hapten, or immobilizing the antibody. To bind the hapten to a surface such as nitrocellulose or plastic it can again first be linked to a protein that binds to these hydrophobic surfaces. However, the protein used for binding the hapten to the surface is different than the protein used for injection of the animal, since the animal also has developed antibodies against the carrier protein used for injection, and only the hapten-specific antibodies are desired for the competitive immunoassay.

17.3.3.2 Bound Hapten Format

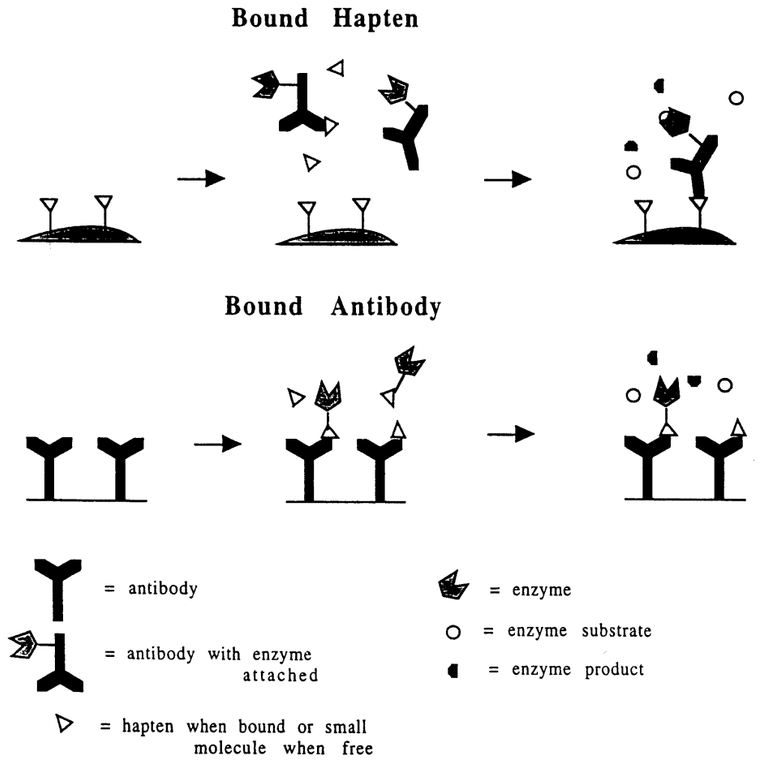
In the bound hapten competitive immunoassay format (Fig. 17-4, top), the protein-bound hapten is first immobilized to some surface, such as plastic, by the same hydrophobic interactions used to bind antibody. Excess material is washed away. Next a competition is created between the protein-bound hapten and the free small molecule in a food extract, both competing for binding to the limited binding sites on the antibody labeled with a bound enzyme. It is important to realize that the free small molecule in the food extract is not completely identical to the immobilized hapten since the latter is covalently linked to a protein. However, if properly designed, the free molecule in the food extract is so chemically similar to the bound hapten that the competition for the limited number of antibody binding sites is nearly equal. The antibody bound to immobilized hapten remains after a subsequent washing step. The more small molecules in the food extract, the more antibody is bound to these free small molecules, and this unbound antibody (and its attached enzyme) will be washed away in the subsequent washing procedure. Finally, the amount of bound antibody is identified by again adding the enzyme substrate and observing the amount of color developed. Therefore, there is an inverse relationship between the amount of small molecules or analyte in the food and the amount of color developed in the final step.

17.3.3.3 Bound Antibody Format

The other variation for a competitive immunoassay is to bind a limited amount of antibody to the plastic and create a competition between hapten bound to enzyme and free small molecules in the food extract (Fig. 17-4, bottom). It is generally believed that this second format is somewhat superior to the first format for sensitivity although it can require the use of more antibody. Again after a washing step, the final procedure is a color development to determine the amount of bound hapten-enzyme. This competitive format also results in an inverse relationship between amount of color and free small molecules in the food extract.

17.3.3.4 Increasing Sensitivity

In order to increase the sensitivity of a competitive immunoassay, the amount of limiting antibody should be reduced. Note that this is exactly the reverse of what one would do to increase the sensitivity of a sandwich immunoassay. Theoretically the most sensitive competitive immunoassay would be between one antibody binding site and one hapten, with either of the two labeled with an enzyme. It is for this reason that the



17-4 figure Competitive immunoassay.

ability to detect the presence of the enzyme is so important for a competitive immunoassay. The more sensitive the system to detect the enzyme, the more sensitive the competitive immunoassay.

17.3.3.5 Similarity to CHARM Tests

The competitive format can be used for any small molecule and binding material. For example, the CHARM tests (5) use immobilized microbial receptors (binding sites on the surfaces of bacteria) rather than antibodies to bind small molecules, and radiolabeled molecules (labeled with carbon 14 or tritium) rather than enzyme labels. However, the format is generally the same as for the bound antibody competitive immunoassay.

17.3.3.6 Cautions in Development

As noted above, great care must be taken in developing a competitive immunoassay since the small molecule is not identical to the hapten, because the hapten is the small molecule linked in some manner to a protein. It is common for the antibody binding site to have a stronger binding affinity to the small molecule plus its linking portion than to the small molecule alone. The antibody binding site will accommodate a molecule of about 1000 g/mol mass, so often this leaves plenty of room to bind both the small molecule and its linking portion. To circumvent this, the linking method for the animal-injected hapten can be carried out in a different manner than the linking of the immunoassay hapten. Hopefully this eliminates the preference for the larger molecule and makes the competition in the immunoassay more equal. Interestingly from a theoretical point of view, a slightly better binding to labeled molecule is preferred to correct for experimental error (6). Concern with interference by derivatives of the target molecule is one of the reasons that it is very important to run controls before and during a competitive immunoassay of a food extract.

Competitive immunoassays also require more care than sandwich immunoassays because materials in the food extract can vary widely and these other components can have an effect on the competition for the antibody binding site. In the sandwich immunoassay, the other materials in the food extract are washed away before the final detection antibody is added, but in the competitive format all food components are present for the only antibody binding step. This is still another reason for examining both positive and negative controls along with any analyzed food sample if the competitive format is used.

17.3.3.7 Standard Curve

If one examines the data for a competitive immunoassay over a wide range of concentrations, the data always fit a sigmoidal curve (Fig. 17-5). Since all types of competitive immunoassays involve a reduction in absorbance with respect to a control (containing no small molecule or analyte), data often are presented as a ratio of sample absorbance to the absorbance of the control. This ratio is the left-side y-axis in Fig. 17-5. As analyte concentration (usually presented on the x-axis using a logarithmic scale) becomes very low, the data curve approaches the maximum absorbance in an asymptotic manner. At the other extreme, very high amounts of analyte prevent antibody binding and the data curve approaches a very low absorbance value in an asymptotic manner. Ideally the low absorbance value is zero, but often it is found experimentally to be somewhat higher than zero. With polyclonal antibodies the main reason for this non-zero bottom limit is that some of the antibodies bind so strongly to hapten that they will not release in the presence of even very large amounts of free small molecules (analyte). The equation that describes the sigmoidal nature of the competitive immunoassay data is:

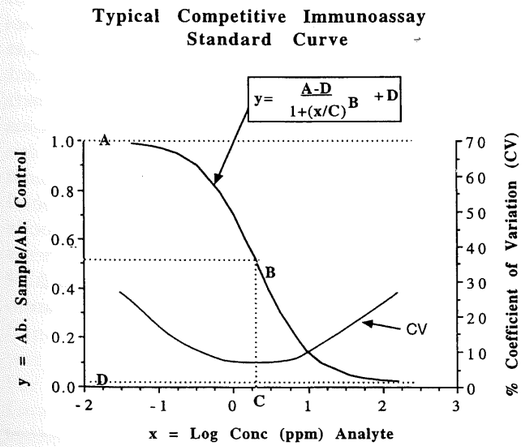
$$y = \left[(A - D)/\left(1 + (x/C)^B\right)\right] + D \qquad [1]$$

where:

A = upper limit on y-axis (1.0 for Fig. 17-5)
D = lower limit on y-axis (0.02 for Fig. 17-5)
C = x coordinate representing y point halfway between A and D, or inflection point of sigmoidal curve [$C = 2(y = 0.51$ and $x = \log 2.0)$ for Fig. 17-5]
B = describes how rapidly the curve makes its transition from A to D ($B = 1.2$ for Fig. 17-5)

The final value, B, often has a magnitude near 1. Smaller values of B indicate a longer, shallower slope while larger values signify a shorter but steeper slope. Note that the value C is a measure of the sensitivity of the assay (2 ppm for Fig. 17-5). C is roughly the concentration of x required to reduce the control absorbance by half. The value of 50% absorbance reduction for different analytes is commonly quoted for immunoassay procedures.

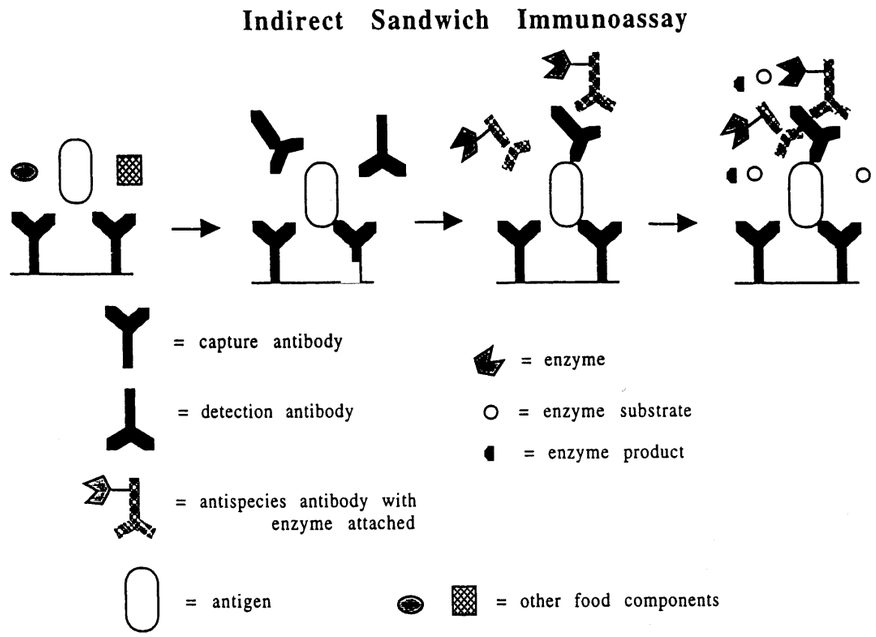
Figure 17-5 also illustrates the error usually found for quantitation of a competitive immunoassay [% coefficient of variation (CV) is solid curve and right-side y-axis]. The smallest CV for quantitation is found close to the C value. As values differ from the C value in either direction, the CV increases. Therefore the most accurate quantitation for a competitive immunoassay experiment occurs near the C value. Two review articles that further discuss data analysis for immunoassays are Tijssen (7) and Rogers (8).



17-5 figure Typical competitive immunoassay standard curve.

17.3.4 Indirect Immunoassays

The sandwich and competitive immunoassays described above both use a **direct** format, meaning that they have some direct way (i.e., enzymes, radiolabel) to measure the amount of antibody or hapten. A popular variation on this format is the **indirect** immunoassay, meaning that they measure the amount of antibody or hapten indirectly, most often with an anti-species antibody. This variation would include indirect sandwich or indirect competitive immunoassays (the indirect sandwich immunoassay is shown in Fig. 17-6). Since antibodies are proteins, they can act as antigens in another animal species. For example, rabbit antibodies injected into a goat can stimulate the goat's immune system to produce goat antibodies that bind to epitopes on the rabbit antibodies. In this way, goat anti-rabbit antibodies can be produced. There are many advantages to these anti-species antibodies. For example, if anti-species antibodies are used in the bound hapten competitive immunoassay format (Fig. 17-4), there is no need to label the rabbit antibody



17-6 figure Example of an indirect sandwich immunoassay.

with an enzyme. After the competition step, excess material can be washed away and goat anti-rabbit antibody labeled with an enzyme can be added to detect the presence of any rabbit antibody. Although this procedure adds a step there can be many advantages. First anti-species antibodies of all types are commercially available from many manufacturers. Also, these anti-species antibodies come with a variety of labels such as different enzymes, radioisotopes, or fluorescent compounds. These different label options become very useful for immunoassay development, or in the use of antibodies for detection in other systems such as examination of tissue under a microscope or proteins separated using some type of chromatography followed by a specific staining procedure. Since the antibody is a very large protein it has many epitopes for attachment of a labeled anti-species antibody. This multiplies the labels per antibody, increasing the ability to detect the antibody, resulting in increasing sensitivity in a competitive immunoassay since less initial competition antibody can be used.

There is still another advantage to anti-species antibodies. Anti-species antibodies also can be prepared in a selective manner. One common use of these selective anti-species antibodies is to differentiate antibody classes. While IgG is the most common class of antibody in human serum (from 8–16 mg/ml), there are other important antibody classes including immunoglobulin M, A, E, and D. These different antibodies classes have different, but consistent amino acid sequences at the bottom of the "Y" portion of the antibody. Of course, anti-species antibodies developed against the different classes of antibody antigens can be prepared. Immunoglobulin E (IgE) can be important to identify because it is associated with allergic reactions in humans. Human IgE antibodies, from subjects with food allergies, are used to determine which food proteins are allergens. Once the proteins and their key epitopes have been identified, animal antibodies can be produced against these proteins. These animal antibodies then are used in immunoassays (most often as sandwich ELISA) to detect allergens in food (9, 10). This can be particularly important when foods are prepared using a variety of different ingredients (11). ELISA test kits (usually sandwich) are available commercially to test for allergens such as egg, peanut, and milk.

17.4 IMMUNOAFFINITY PURIFICATION

Besides the use of antibodies in immunoassays as described above, often antibodies are used in food analyses as complements to other analytical methods. This is due to the remarkable specificity of antibodies and their strong binding to antigen. The most common example of this is **immunoaffinity** purification, which is an antigen capture technique. Basically the antibody is immobilized on some support, most often using a covalent linking method so that there is no concern with "bleeding" of the antibody in later steps. The antibody can be bound to a solid phase such as agarose or silica gel. These antibody-bound solid phases can be used later for purification of antigen via a chromatography method or by the use of these phases on the surface of magnetic beads that are separated using a magnet. A simple purification sequence would involve exposing the antibody-bound solid to a food extract to first bind antigen, then washing the solid phase free of all unbound material, and finally releasing the pure antigen. Even though antibodies have such remarkably strong binding constants, they can be treated to release antigen by simple procedures such as changes in pH or solvent. Since the antibody is a protein, pH changes or solvent changes result in denaturation that changes the conformation of the binding site, releasing antigen. If these changes are carefully selected, denaturation can be reversed by reestablishing moderate conditions so that the valuable antibody-bound solid phase can be reused repeatedly. Of course for sensitive antigens, like enzymes, these elution conditions also can be a concern.

These immunoaffinity purification procedures have been used for small molecules like toxins (e.g., aflatoxins) and even materials as large as cells. Different microorganisms contain unique cell surface antigens that can be selectively bound to aid in purification and differentiation.

In its simplest form, antibody capture of antigen can involve a simple precipitation. Since all antibodies have at least two identical binding sites, they can crosslink epitopes from two identical antigens. If other antigen epitopes are further crosslinked by different antibodies, a large, insoluble network can result which is seen as a precipitate. Of course this precipitation will only work for antigens with more than one epitope. However, these precipitation reactions have been used widely for protein and cell identification.

17.5 APPLICATIONS

Immunoassays are a well-developed area in food analysis and there are several good textbooks available. For all sorts of laboratory techniques, Harlow and Lane (12) wrote one of the best books. The theory of immunoassays is well handled in several books (13, 14). There are even entire journals such as *Food and Agricultural Immunology* devoted to describing methods for preparing food immunoassays.

Because of the simplicity, sensitivity, and specificity of immunoassays, they are used widely as screening tests for pesticide (15) and drug residues (5) in food (see Chapter 19). Besides chemical analysis, immunoassay techniques are used in microbiology to rapidly detect food-borne pathogens (16) and especially bacterial toxins (17). Immunoassays also are commonly used for meat species identification (18). Since immunoassays can easily be developed to detect small amounts of specific proteins, they are among a number of methods used to detect genetically modified organisms in foods (19) (see Chapter 18). With small solutions and repetitive procedures, immunoassays are being automated for higher analytical throughput. The push for automation of immunoassays comes mainly from their use for medical analyses (20).

17.6 SUMMARY

Almost any organic molecule in food can be determined using immunoassays. The remarkable selectivity and specificity of these assays are the result of the strong binding between antibodies and their antigens. While the precise protocols of immunoassays can vary a great deal, all immunoassays use either a direct detection or a competitive format. The competitive format is the only one that can be used for quantification of small (about 1000 g/mol or less) molecules. Key to the competitive format is the ability to separate and detect free and antibody-bound antigen by labeling either the antigen or the antibody. The most common labels used for food immunoassays are enzymes and the most common enzyme used is horseradish peroxidase. These immunoassays are widely known as ELISA methods. In a competitive ELISA with enzyme-derived color development, the more antibody bound molecules (analyte) in your food sample the less color develops.

Food immunoassays can be prepared using very simple and rapid formats, making them ideal for kits used in the field. Such kits are commonly used for pesticide or residue testing at parts per million or lower levels of detection. While every effort is made to control the specificity of these field tests, they can suffer from false positives and false negatives. For this reason, immunoassay kits are used most often as rapid screening tests, while residues in food samples that test positive by immunoassay are confirmed using another, more laborious method.

Besides being the required constituent in immunoassays, antibodies also can be used to purify specific compounds in food for other analysis methods. These immunoaffinity purification methods allow for rapid purification of analytes from complex food matrices. Also, if properly handled these purification materials can be reused.

17.7 STUDY QUESTIONS

1. What is the relationship between an antigen and an antibody?
2. What is the difference between monoclonal and polyclonal antibodies?
3. All immunoassays have two conditions that they must satisfy; what are they?
4. What is a hapten?
5. Two common immunoassays are the sandwich assay and the competitive assay. Which molecules are best detected by each? Why?
6. What factors would you change to increase the sensitivity of a sandwich immunoassay and a competitive immunoassay?
7. Explain why the concentration of antigen required to reduce the absorbance by 50% (usually very close to the value C for the equation of the sigmoidal curve) is such a useful value to determine for a competitive immunoassay.
8. Give the advantages and disadvantages of establishing an indirect format for immunoassays.
9. Describe, in general terms, how you would use immunoaffinity purification to isolate a protein for which you have developed antibodies.
10. Give four common applications of immunoassays in food analysis.
11. All commercial potatoes contain the toxic glycoalkaloids α-solanine and α-chaconine. Both of these glycoalkaloids have the same large alkaloid portion, known as solanidine. Therefore polyclonal antibodies can be developed in rabbits against solanidine by chemically linking it to a foreign protein (foreign to the rabbit) and injecting the protein-bound hapten (solanidine linked using a succinic acid derivative) into rabbits. The antibodies that develop in the rabbit against the hapten bind to the alkaloid portion of both toxic glycoalkaloids.

To develop an appropriate competitive ELISA, solanidine is again linked to a protein, but this time a different protein, and this conjugate is used to coat plastic microtiter plates. After excess conjugate is washed away the plates are ready for the competitive ELISA procedure.

The glycoalkaloids in potatoes are extracted with methanol and this extract is further diluted with water for use in the ELISA procedure. A standard curve is prepared by diluting standard solutions of α-chaconine at low, medium, and high concentrations with similar aqueous methanol solutions. In addition a negative control is prepared using methanol and water at similar concentrations to the diluted potato extracts and standards, but without any glycoalkaloid present. Now the various extracts, standards, and negative controls are placed in individual wells with equivalent amounts of diluted rabbit serum containing the polyclonal antibodies. After incubation for 30 min at room temperature, all of the wells on the plate are again washed. Next a solution of commercially available goat anti-rabbit antibody conjugated to peroxidase is added to each well. After another 30 min incubation the wells are again thoroughly washed.

Finally phenylenediamine substrate solution is added to each well along with peroxide and again the plate is incubated for 30 min. After 30 min the plate is rapidly read (in under one minute) using an ELISA plate reader. The wells all contain differing amounts of yellow color.

a. Tomatidine is a glycoalkaloid found in tomatoes and contains the alkaloid portion tomatine. Would the polyclonal antibodies detect tomatidine?
b. Why is the protein that the hapten is attached to different for the ELISA procedure than for the injection?
c. Is the ELISA protocol direct or indirect?
d. Which wells would you expect to contain the most color, standards, potato extracts, or negative controls?
e. Would you be concerned if a potato extract gave almost no color at the end of the ELISA procedure?

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