1. The first step is to identify the problem or question that needs to be answered. This involves understanding the context and the specific requirements of the task.

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**APPLICATIONS OF MACROPOROUS HYDROPHOBIC CLOTHS
AS SOLID PHASES FOR ENZYME IMMUNOASSAY**

by

Burton W. Blais, B. Sc., M. Sc.

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy**

**Department of Biology
Carleton University
Ottawa, Ontario
April 9, 1990.**

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The undersigned hereby recommend to the
Faculty of Graduate Studies and Research
acceptance of this thesis, entitled,
'Applications of macroporous hydrophobic cloths
as solid phases for enzyme immunoassay',
submitted by Burton W. Blais, B. Sc., M. Sc.,
in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy.

Chairman, Department of Biology

Thesis Supervisor

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For Lisa

**"Whoso loveth instruction
loveth knowledge"
-Proverbs 12:1.**

ABSTRACT

This thesis examines the application of macroporous hydrophobic cloths, such as polyester cloth, as adsorbents of immunoreactants for use in solid phase enzyme immunoassays (EIA). The large surface area of polyester cloth permitted the rapid quantitative assay of antibodies. Salmonella lipopolysaccharide (LPS)-coated cloth was instantaneously exposed to anti-Salmonella antibody, and the captured antibody was detected by an antiglobulin-peroxidase conjugate which produced an enzyme signal on the cloth that was proportional to the antibody concentration.

The capacity of the cloth to accommodate a large volume of antibody sample in close contact with the immobilized LPS antigens was exploited in the rapid assay of anti-Salmonella antibodies in chicken egg yolk, a highly viscous sample in which the rate of antibody diffusion is limited by the viscosity. The assay revealed a considerable amount of fluctuation in the antibody level in eggs. This assay can potentially be used to monitor the level of Salmonella contamination in poultry rearing facilities.

To immobilize a hydrophilic polysaccharide antigen derived from Salmonella LPS, polyester cloth was activated with cyanuric chloride, which allowed for the covalent attachment of the polysaccharide. This broadens the variety of antigens which can be immobilized onto the cloth surface.

Heating Salmonella cells in the presence of

ethylenediaminetetraacetate (EDTA) dissociated its antigens into non-sedimentable forms. This allowed for the extraction of the antigens from solid-rich samples into a supernatant after centrifugation. The extracted antigens exhibited rapid immunoreactions with anti-Salmonella antibodies adsorbed onto polyester cloth. The macroporosity of the cloth permitted the concentration of large volumes of the extracted antigens on antibody-coated cloth by rapid filtration, thus increasing the effective sensitivity of the EIA.

Polymyxin-coated polyester cloth was found to capture Salmonella LPS antigens for their EIA, eliminating the need for expensive coating antibody. Heating Salmonella cells in deoxycholate greatly enhanced the interaction of the LPS antigens with polymyxin-cloth. A 24 h procedure for the detection and semi-quantitation of salmonellae in foods was developed using a combination of enrichment culture and a dot blot assay on polymyxin-cloth.

Salmonella LPS-coated cloth was used in a simple procedure for the rapid affinity purification and biotinylation of anti-Salmonella antibodies on the solid phase. The biotinylated antibodies were useful as an immunoreagent for EIA.

The methodology developed in the present thesis should be applicable to the assay of a variety of antibodies and antigens.

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ABBREVIATIONS

A ₃₇₀	absorbance at 370 nm
A ₄₁₄	absorbance at 414 nm
Antibody-cloth	antibody-coated cloth
ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline -6-sulfonic acid)
°C	degrees celcius
CEIA	cloth-based enzyme immunoassay
E	enzyme
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay(s)
g	gram
h	hour(s)
l	liter
LPS	lipopolysaccharide(s)
mg	milligram (10 ⁻³ g)
ml	milliliter (10 ⁻³ l)
µg	microgram (10 ⁻⁶ g)
µl	microliter (10 ⁻⁶ l)
min	minute(s)
PBS	0.01 M phosphate-buffered (pH 7.2) - 0.85% NaCl
PBST	PBS containing 0.05% Tween 20.
Polymyxin-cloth	polymyxin-coated cloth
TMB	3,3',5,5'-tetramethylbenzidine

GENERAL INTRODUCTION

In the mid-1950s Solomon Berson and Rosalyn Yalow were investigating the cause of maturity-onset diabetes. Their hypothesis, that a hormonal insufficiency resulted when diabetic individuals eliminated insulin too rapidly from their bodies, was tested by studying the metabolism of radiolabelled insulin injected into normal and diabetic subjects (Yalow, 1978). Contrary to their expectations, they found that radiolabelled insulin was retained longer in the blood of diabetics than non-diabetics. Further studies revealed that the retention of insulin in diabetic individuals was due to the binding of anti-insulin antibodies, probably produced as a result of the repeated administration of exogenous insulin during routine treatment of the disease. Carrying their experiments yet further, Berson and Yalow then observed that the binding of radiolabelled insulin to a fixed amount of anti-insulin antibody is an inverse function of the amount of unlabelled insulin present, an observation that was to become the basis for the sensitive in vitro radioimmunoassay (RIA) of insulin (Berson and Yalow, 1960), and later, a veritable plethora of other antigens and haptens. In recognition for her role in the development of the RIA technique, Dr. Yalow was awarded the Nobel prize for medicine in 1977. Thus, the foundation

for modern immunoassay technology, that auspicious combination of medicine and biotechnology which deals with the identification and quantitation of antigens and haptens bearing on human, animal and plant diseases, was established as a result of investigations whose original course lay elsewhere.

The principle of antigen (or hapten) detection by RIA is exquisitely simple: a known amount of radiolabelled antigen standard and an unknown amount of unlabelled antigen present in a test sample are mixed together with a fixed amount of antibody to the antigen, and then incubated. Since both labelled and unlabelled antigen molecules will compete for binding sites on the antibody, the amount of sample antigen is estimated by measuring the radioactivity bound to the antibody, which is inversely related to the amount of sample antigen (Yalow, 1978). In order to measure the amount of antigen bound to the antibody, it is necessary to separate antibody-bound antigen from free antigen. This can be accomplished by one of several methods, including immunoprecipitation of antibody-bound antigen by use of an anti-globulin reagent (McGuigan, 1968), the removal of free antigen by adsorption onto charcoal (Newsome and Shields, 1981), or by employing a solid-phase RIA format where the

antibody is first immobilized onto particles (e.g., Sephadex beads) which can then be separated by centrifugation (Levitt and Schmitt, 1976). Whatever the design of the assay, the main advantages of RIA are its ability to exploit the specificity of the antibody-antigen reaction and the extremely high sensitivity which can be achieved, with some assays permitting the detection of picogram quantities of antigen (Aherne, 1985). However, the disadvantages of RIA cannot be overlooked. These include the requirement for expensive instruments to measure radioactivity, the short shelf-life of the radiosotopes used to label reagents, the potential health hazard involved in the handling of radioisotopes, and the waste disposal problem which they create (Tijssen, 1985).

About ten years after the pioneer work of Berson and Yalow in the field of RIA came the development of the enzyme immunoassay (EIA) technique, in which immunoreagents (antibodies or antigens) are labelled with an appropriate enzyme instead of a radioisotope. Although the use of enzyme-labelled immunoreagents, particularly antibody-enzyme conjugates, evolved fairly independently (originally out of the need to localize antigens in histological preparations), the impressive developments taking place concurrently in the

field of RIA immunodiagnostics quickly prompted researchers to apply these enzyme-labelled reagents in the assay of antigens using strategies similar (though not identical) to those employed in RIA. In EIA, both the specificity of antibodies and the amplifying (catalytic) properties of enzymes are exploited to give a highly specific and sensitive assay for the detection of antigens. Thus, all EIA have two essential features: the reaction of a specific antibody with an antigen, and the detection of this immunoreaction using an enzyme to which one of the reagents (antibody, antigen or hapten) is conjugated as an indicator. The use of enzymes instead of radioisotopes as indicators in the assay of antigens not only eliminates the disadvantages of RIA, but also provides some important additional improvements (Tijssen, 1985): (1) potentially very high sensitivities can be achieved, since an amplified response is inherent in the ability of enzymes to catalyze the conversion of many substrate molecules to detectable product; (2) the availability of a broad variety of substrates (e.g., chromogenic, fluorogenic and chemiluminescent) for the most commonly used enzymes allows for either visual (qualitative) or instrumental (quantitative) tests; and, (3) reagents and equipment

required for EIA are relatively inexpensive and readily available.

EIA has been successfully applied to the detection of a wide variety of substances that are antigenic (e.g., proteins) or can be rendered antigenic (e.g., haptens), from specific cell surface components of bacterial pathogens (Perera et al., 1983) to environmental pollutants (Van Emon et al., 1986). They have been equally useful for the detection of antigen-specific antibodies in sera or secretions of animals and humans for the immunodiagnosis of infectious diseases (Voller et al., 1976). Table 1 summarizes some of the areas in which EIA have found extremely useful applications. Depending on the specific application, EIA can be adapted for use in either a large central laboratory, where facilities are available for the automation of routine tests when large numbers (i.e., several hundred) of samples must be processed on a regular basis, or they can be designed for use under field conditions (e.g., small clinics or wherever on-site testing is required), where only small numbers (i.e., a few dozen) of samples need to be processed.

Although many different types of EIA procedures have been developed, the most popular and straightforward are the

Table 1. Examples of applications of the EIA technique.

Target molecule (analyte)	Applications
Antibodies	<ul style="list-style-type: none"> -Immunodiagnosis of viral, bacterial and fungal diseases of humans and animals -Screening blood supplies for previous exposure to transmissible agents such as viruses
Antigens	<ul style="list-style-type: none"> -Detection of microbial antigens in clinical specimens -Detection of bacterial and fungal toxins -Measurement of polypeptide hormones in blood
Haptens ^a	<ul style="list-style-type: none"> -Detection of environmental pollutants such as pesticides and industrial waste products -Detection of drugs and their metabolites in body fluids

^a A hapten is any low molecular weight molecule that in itself is non-antigenic but when attached to a larger molecule, such as a carrier protein, can induce the formation of hapten-specific antibodies.

non-competitive solid phase assays which employ an immunoreactant (antibody or antigen) immobilized on a solid phase for the immunospecific capture of its corresponding antigen (or antibody) from a sample (Engvall, 1980). The antigen or antibody captured on the solid phase is then detected using a specific antibody-enzyme conjugate system, and the bound enzyme activity (which is a quantitative function of the amount of antigen or antibody captured on the solid phase) is measured by incubation with a suitable substrate yielding a detectable product (Fig. 1).

Alternatively, competitive solid phase assay formats using either enzyme-labelled antibody or enzyme-labelled antigen (or hapten) can be employed for antigen detection.

Depending on the type of conjugate used, competitive assays are based on either (1) the inhibition by free antigen (or hapten) present in the test sample of the reaction of enzyme-labelled antibodies with antigen (or hapten) immobilized on the solid phase, or (2) the competition of enzyme-labelled antigen (or hapten) with the antigen (or hapten) present in the test sample for antibody immobilized on the solid phase. In either case, the amount of enzyme-labelled immunoreagent bound to the solid phase is inversely related to the concentration of free antigen (or hapten) in

the test sample (Fig. 2).

The choice of an enzyme for use in EIA is governed by several criteria (O'Sullivan and Marks, 1981): (1) low-cost purified homogeneous enzyme preparations should be available; (2) the enzyme preparation should possess a high specific activity; (3) the enzyme should possess a functional group through which it can be cross-linked to antibodies or antigens to produce stable conjugates, with minimal loss of enzyme and immunological activity; and, (4) a simple method for assaying enzyme activity should be available. Of the enzymes which meet these requirements, the most commonly employed in EIA are alkaline phosphatase, horseradish peroxidase, B-galactosidase, urease, and glucose oxidase.

Enzymes can be conjugated to antibodies, antigens or haptens by a variety of methods. The choice of a cross-linking method depends mainly on the type of functional groups available on these molecules. Glutaraldehyde, a homobifunctional aldehyde, has long been recognized as an effective agent for cross-linking molecules via their free amino groups (Habeeb and Hiramoto, 1968), and has been successfully employed in the preparation of antibody-alkaline phosphatase conjugates (Avrameas, 1969). The

Fig. 1. Non-competitive solid phase EIA. (A) Detection of antigens ('sandwich' format). Antibodies specific for the antigen of interest are immobilized on the hydrophobic solid phase, usually by hydrophobic interaction, which is then incubated with the test sample (e.g., a bacterial culture) containing the antigen. The solid phase is then washed with buffer to remove unbound sample components, and the captured antigen is detected by incubation with an antigen-specific antibody-enzyme conjugate (the antigen becomes 'sandwiched' between the capture antibody and the conjugate), followed by a wash with buffer and incubation with enzyme substrate. Note that the presence of at least two epitopes on the antigen is required for both antigen capture and its detection using conjugate. (B) Detection of specific antibodies. The solid phase is coated with antigen and then incubated with a test sample (e.g., serum) containing the antibodies to be detected. The solid phase is then washed with buffer, leaving only the antigen-specific antibodies bound. These antibodies are then detected by incubating the solid phase with an anti-globulin antibody-enzyme conjugate, followed by another wash with buffer, and finally, incubation with enzyme substrate.

(A)

(B)

Fig. 2. Competitive solid phase EIA. (A) Detection of antigen using enzyme-labelled antibody. Antigen is immobilized on the solid phase, which is then incubated with a mixture of the test sample containing the free antigen and a fixed amount of enzyme-labelled antibody. The solid phase is then washed with buffer and assayed for bound conjugate by incubation with enzyme substrate. (B) Detection of antigen using enzyme-labelled antigen. An antibody specific for the antigen of interest is immobilized on the solid phase, and then incubated with the test sample containing free antigen, followed by a fixed amount of enzyme-labelled antigen. The solid phase is then washed and assayed for bound conjugate as above. In both cases, the amount of conjugate bound to the solid phase is inversely related to the concentration of antigen in the test sample.

(A)

(B)

precise mechanism of the conjugation reaction is not known; however, it is believed that polymerized forms of glutaraldehyde (e.g., di- and tri-mers) are involved. Whatever the mechanism, the reaction of amino groups with the aldehyde produces a very stable link between the conjugated molecules (Habeeb and Hiramoto, 1968).

On the other hand, glutaraldehyde is of limited use for the conjugation of horseradish peroxidase to antibodies, since this enzyme has few free amino groups available on its surface for reaction with the aldehyde. Therefore, the periodate oxidation method of Nakane and Kawaoi (1974) is widely used for the conjugation of horseradish peroxidase to antibodies. In this method, the carbohydrate residues of this glycoprotein enzyme are oxidized with periodate to generate aldehydes, which can then be reacted with a free amino group on an antibody molecule to give a link which is a Schiff base. The link is then stabilized by reducing the Schiff base with borohydride. This method of coupling an enzyme to an antibody is illustrated in Fig. 3.

Other bifunctional cross-linking agents which are useful in the preparation of antibody- or antigen-enzyme conjugates include p-benzoquinone, which can react with either amino or sulfhydryl groups, and m-maleimidobenzoyl-N-

hydroxysuccinimide ester, a heterobifunctional reagent which reacts sequentially with amino and then sufhydryl groups (Tijssen, 1985).

Fig. 3. Conjugation of enzyme to antibody by the periodate oxidation method.

The usefulness of an enzyme as an indicator for EIA ultimately depends upon the availability of a suitable substrate system which permits its sensitive and convenient assay. A variety of chromogenic substrates have been

developed for each of the different enzymes used in EIA. For alkaline phosphatase the most popular chromogenic substrate is p-nitrophenyl phosphate, a colourless compound which is cleaved by the enzyme at alkaline pH to give a highly coloured soluble product, p-nitrophenol, which can be readily assessed visually or assayed by spectrophotometry. Considerable progress has also been made in the development of substrates for the extremely sensitive assay of alkaline phosphatase, such as 4-methylumbelliferyl phosphate, a fluorogenic substrate which greatly increases the sensitivity of EIA using this enzyme (Volken and Leister, 1982). However, the use of such substrates requires special instruments (fluorometers) for the detection of the fluorescent products, and may therefore not be practical for all situations (e.g., during testing under field conditions). Horseradish peroxidase, which catalyzes the oxidation of various hydrogen-donating compounds by hydrogen peroxide, can be assayed using a number of chromogenic hydrogen donors such as 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and o-phenylenediamine (OPD) (Tijssen, 1985). Unfortunately, many of these compounds are mutagenic, and hence, hazardous to the user. Another chromogenic hydrogen-donating compound

which is becoming increasingly popular is 3,3',5,5'-tetramethylbenzidine (TMB), which is non-mutagenic (Bos et al., 1981) and which provides greater sensitivity in EIA than the other chromogens (Hosoda et al., 1986). Although several fluorogenic and chemiluminescent hydrogen donors are available (Whitehead et al., 1983), they require special instruments and are therefore of limited use for EIA under field conditions.

The immobilization of immunoreactants on a solid phase is one of the most crucial aspects which must be addressed when designing a solid phase EIA. The use of an appropriate solid phase not only simplifies the manipulations (handling and washing) required to separate free antibody or antigen from immunoreacted components, but also will play an important role in dictating the success or failure of an assay. Immunoreactants (antibodies and some antigens) can be immobilized by covalent binding to a wide variety of surfaces, such as glutaraldehyde-treated HCl-activated nylon beads (Perera et al., 1983), cyanogen-activated polyhydroxy supports (e.g., Sephadex beads) (Yolken, 1982), or a periodate-oxidized polyester film (Sarkar and Mandal, 1985). However, the most popular solid phases in use are non-porous hydrophobic plastic surfaces, such as multi-well polystyrene

microtiter plates, tubes or beads made of polystyrene (Ch'ng et al., 1985), polyvinyl chloride, and polymethacrylate (Tijssen, 1985). Antibodies and some antigens are usually immobilized on such surfaces by hydrophobic interaction between sites on the immunoreactant molecule and the hydrophobic plastic surface. Other solid phases to which immunoreactants have been adsorbed include filter paper (Hamblin and Hedger, 1982) and microporous surfaces such as membranes of nitrocellulose (Brower et al., 1985), nylon (Tijssen, 1985) and polyvinylidene difluoride (Oprandy and Sippel, 1989).

Since in non-competitive solid phase EIA the immunoreactions occur on the heterogeneous phase, the rate of antibody-antigen interactions will be lower than that in free solution. The interactions in these solid phase EIA are limited by diffusion rate (i.e., the rate at which antigen or antibody in a liquid sample diffuses to the immunoreactant immobilized on the solid phase). Typically, immunoreactions performed on non-porous solid phases having a small surface area (such as the wells of a microtiter plate) require in excess of 1 hour to complete (i.e., attain equilibrium), whereas such reactions in free solution are complete within a few minutes (Tijssen, 1985). This

disadvantage not only increases the time required to complete an assay, but also limits the applications of EIA.

The kinetics of antibody-antigen interactions in solid phase EIA can be significantly improved by using a microporous (e.g., nitrocellulose membrane) or particulate (e.g., micro-beads of polyacrylamide or agarose) solid phase having a high surface area for immunoreactant immobilization (Tijssen, 1985). However, these solid phases also have significant drawbacks. Microporous membranes such as nitrocellulose and nylon are brittle, making them difficult to handle; extremely thin, limiting the sample volume that can be applied per unit area (e.g., only a few microliters can be accommodated by a 25 mm² segment); and, they have a tendency to non-specifically adsorb immunoreagents such as conjugate (Spinola and Cannon, 1985), causing high background enzyme signals (this problem is usually alleviated by blocking non-specific binding sites on the membrane with a protein such as bovine serum albumin, thus adding to the time and expense of the assay). The use of particulate solid phases is also limited in that they usually require the covalent immobilization of immunoreactants, a procedure which can be time consuming, expensive and of uncertain reproducibility. They are also

difficult to handle and wash between each immunoreaction, requiring special devices for transferring and containing the particles. Thus, there is a great need for a more efficient, practical and versatile solid phase in order to broaden the applications of EIA as a diagnostic tool. The present thesis addresses this need by examining the advantages and applications of a new type of solid phase, macroporous hydrophobic cloths, as adsorbents of immunoreactants for use in EIA.

Previously, my M. Sc. thesis work on the use of macroporous hydrophobic cloths as immunoabsorbents for EIA has shown that several different types of cloths can adsorb sufficient amounts of antibodies to allow for the capture and sensitive detection of bacterial and viral antigens using specific antibody-enzyme conjugates (Blais, 1986). Cloths composed of synthetic polymer fibers of polyethylene terephthalates (polyester), propylene, or amide (nylon) gave excellent results in a cloth-based EIA (CEIA) for the detection of Brucella abortus cell surface antigens, with the polyester cloth performing best. When applied in a CEIA for the detection of Brucella antigens in enrichment cultures of various mock-infected bovine tissues, polyester cloth coated with diluted bovine serum containing anti-

Brucella antibodies enabled the efficient capture of B. abortus and B. melitensis antigens, which were then detected on the cloth using an anti-Brucella antibody-enzyme conjugate (Blais et al., 1989). The basic structure of polyester cloth is shown below.

Although not previously demonstrated, nor fully exploited, the attributes of cloths (e.g., polyester cloth) which can make them efficient and highly versatile immunoabsorbents for EIA are considerable. These attributes reside chiefly in the macroporous nature of the cloths, which provides for: (1) a large surface area for immunoreagent immobilization; (2) the ability to accommodate a relatively large volume of sample (per unit area) which is held in intimate contact with the immunoabsorbent surface; and, (3) excellent filtration characteristics (which allows for simpler and more efficient washing procedures). Furthermore, such cloths are readily available and inexpensive. It is surmised that the first two attributes

should result in excellent association kinetics (i.e., faster immunoreactions), thus increasing the flexibility of the CEIA method and broadening its applications. For example, this advantage, in combination with the third attribute of cloths, should allow for the rapid concentration of dilute antigens on antibody-coated cloth by filtration, thereby increasing the sensitivity of the antigen CEIA by taking full advantage of the entire sample volume available. The following chapters will show how these advantages can be applied to the detection of specific antibodies and antigens by CEIA. As an example, a Salmonella antigen-antibody system will be examined.

Members of the genus Salmonella, the major cause of human enteritis worldwide, are usually spread to their human hosts by contaminated foods, milk and water. One of the major sources of salmonellosis in man is poultry meat, which has a high incidence of Salmonella contamination due to the excellent environment provided by the intestinal tract of chickens for the growth of the organisms and the ease with which they can be transmitted among birds maintained in rearing facilities (Lee et al., 1983). Furthermore, the modern practice of raising young chicks in a sterile environment has rendered them increasingly susceptible to

Salmonella infection, since under such circumstances they fail to acquire the natural intestinal microflora necessary for protection against invading pathogens (Pivnick and Nurmi, 1982). The result is that, in Canada, the incidence of Salmonella contamination in chicken carcasses has grown from 23% in 1974 to 61% in 1983 (Stavric, 1987). Since clinical symptoms are rarely manifested in infected chickens (there are some exceptions, such as Salmonella pullorum infections), the carriers are difficult to identify (Thain and Cullen, 1978). Over the past decade or so there has been a growing consumer trend throughout the western world toward replacing the traditional red meats (e.g., beef), which are high in saturated fats and cholesterol, for the generally healthier white meats such as poultry. This may partially account for the large increases in the incidence of human salmonellosis seen worldwide in recent years (Dixon, 1986; Stavric, 1987). Thus, the Salmonella problem has become an important public health issue, and the need for stricter measures to minimize the exposure of humans (and animals) to contaminated foods is now widely recognized. One means of accomplishing this involves the direct detection of salmonellae in foods.

The current standard in North America for the detection

of salmonellae in foods is the pure-culture technique. The established procedure requires a series of culture steps followed by biochemical and serological identification, and can be summarized as follows (Andrews, 1985): (1) pre-enrichment of the sample in a nutrient-rich broth to allow for recovery of potentially damaged cells, followed by (2) enrichment in a selective broth to suppress the growth of non-Salmonella organisms, then (3) isolation of pure cultures of Salmonella by streaking onto selective agar plates, and (4) biochemical and serological characterization to confirm the identity of any isolates. The entire procedure is costly and time consuming, requiring 5-7 days to complete.

There have been many reports in the literature on the development of more rapid methods of detection involving shorter culture steps and more sensitive techniques for the identification of Salmonella organisms in enrichment cultures rather than on plates. The latter include a fluorescent antibody technique (FAT) (Gibbs et al., 1972), and a selective motility technique (SMT) (Holbrook et al., 1989), both of which permitted the detection of salmonellae within 2-3 days. In the FAT, salmonellae present in enrichment cultures are fixed onto a slide which is then

incubated with a fluorescein-labelled anti-Salmonella antibody, and the slide is then examined for the presence of fluorescent cells by fluorescence microscopy. The SMT, which only detects motile salmonellae, is based on the migration of the cells from an enrichment broth into tubes containing selective and diagnostic media. A similar type of test which is now commercially available in a kit form (the 1-2 TEST, BioControl Systems, Inc.) involves a single-use plastic device with two compartments-an inoculation chamber containing a semi-solid selective enrichment medium, and a 'motility' chamber containing a semi-solid medium to which an anti-Salmonella antibody is added, and into which motile salmonellae migrate to form a visible precipitin line upon reaction with the antibody.

The past decade has also seen the emergence of DNA-DNA hybridization tests, which consist of immobilizing the genomic DNA of the target organism (usually on a membrane filter) and detecting nucleotide sequences that are unique to the organism by hybridization with a specific ^{32}P -labelled DNA probe, followed by autoradiography to detect the probe (Fitts, 1985). Tests for the sensitive detection of salmonellae in enrichment cultures using this technology are now commercially available (e.g., the Gene-Trak test,

Integrated Genetics). However, these tests suffer from several disadvantages, including those involved in the use of a radioisotope as the indicator (e.g., short shelf-life of the radioisotope, potential health hazard, sophisticated instruments needed for detection), the time required to complete a test and the high cost involved. Alternatively, DNA-DNA hybridization tests employing biotin-labelled DNA probes in combination with an avidin-enzyme conjugate (which binds to the biotinylated probe, thus permitting its detection) have been developed in order to eliminate the need for radioactive labels and to simplify the visualization procedure (Leary *et al.*, 1983). These DNA-DNA hybridization methods may initially require a considerable effort to identify the appropriate nucleotide sequences needed to define a specific probe. When the amount of genetic material present in a sample is insufficient to allow for detection by these tests, target DNA sequences can be amplified to detectable levels by using the polymerase chain reaction (PCR), a recently developed technique which has been successfully applied to the detection of minute quantities of DNA from many bacterial, fungal, and viral pathogens (Kwok and Sninsky, 1989). However, the PCR method alone cannot distinguish between

viable and non-viable cells in a sample, and thus may not be suitable for direct assays of viable pathogens in foods.

Although all of the above methods are sufficiently sensitive to permit the detection of salmonellae in early enrichment cultures, their application can be costly, time consuming and technically demanding. On the other hand, the advantages of EIA are particularly well-suited for this task, and there have been several reports of EIA procedures for the detection of Salmonella antigens in cultures after only 2 days' enrichment (Ibrahim and Lyons, 1987; Swaminathan et al., 1985).

The requirement for amplification of the salmonellae by culture methods is practically indispensable, since the numbers of organisms present in contaminated food samples can be extremely low (e.g., <10 cells/25 g sample; Andrews, 1985), and no assay method currently available (including EIA) can detect such low numbers directly. Detection methods involving the initial culture of microorganisms as currently practiced provide purely qualitative results, since it is impossible to quantitatively determine the original level of contamination in a sample after enrichment in liquid media. Since the extremely high prevalence of salmonellae in poultry meat is now recognized (Stavric,

1987), merely detecting their presence may no longer provide sufficient information for effective management of the problem. More relevant information could be gained from a quantitative (or semi-quantitative) method able to discriminate between high and low levels of contamination, thus allowing for the identification of grossly contaminated samples which may pose a more immediate threat to public health. The development of such a semi-quantitative procedure incorporating the unique advantages of the CEIA method is one aspect of the Salmonella problem that will be addressed in forthcoming chapters.

The purpose of this thesis is two-fold: (1) to demonstrate the unique advantages of the CEIA method as a diagnostic tool for the detection of specific antibodies and antigens, and (2) to exploit these advantages in the development of simple, rapid, sensitive and economical procedures for the monitoring of Salmonella contamination in foods and other samples.

CHAPTER 1

USE OF A HYDROPHOBIC CLOTH FOR ENZYME IMMUNOASSAY

ABSTRACT

As an immunoabsorbent for enzyme immunoassay, a macroporous polyester cloth was superior to the conventional non-porous microtiter plate in that it provides a much larger surface area for immunoreactions (thus, yielding faster reactions), can accommodate a larger volume of sample per unit area (maximizing the capture of sample immunoreactants), and permits easier washing between each immunoreaction step. These attributes should enable polyester cloth to provide rapid and simple field assays for a variety of antigenic substances.

INTRODUCTION

The definitive diagnosis of infectious diseases requires the cultivation of the infectious agent in an in vitro system or laboratory animal, which is a laborious, time consuming and expensive approach. Alternatively, the exposure of humans or animals to infectious agents can be determined with great ease by simply assaying their serum for the presence of specific antibodies. In principle, infecting pathogens (whether they be fungi, bacteria, or viruses) will elicit an antibody response from the host, and the detection of specific antibodies in significant

quantities is highly suggestive of disease. Specific antibodies can be measured by several methods, including the classic tube agglutination and complement fixation tests (Richter, 1982), or by enzyme immunoassay (EIA) using antigens immobilized on a solid phase for the capture of specific antibodies from the test sample, followed by their detection with an antibody-enzyme conjugate (Tijssen, 1985). Of all the methods available, EIA is by far the most sensitive for the detection and quantitation of antibodies, and many clinical laboratories throughout the world currently employ EIA tests for the immunodiagnosis of a wide variety of infectious diseases.

The most commonly used EIA procedures employ non-porous polystyrene or polyvinyl microtiter plates for the immobilization of antigens such as viral proteins (Chu et al., 1985) or bacterial lipopolysaccharide (Boraker et al., 1981) in the assay of specific antibodies. However, because of their limited surface area such non-porous solid phases cannot adsorb large quantities of immunoreactant, resulting in slower reaction kinetics (necessitating longer immunoreactions) and saturation of the immobilized antigens at high antibody concentrations (affecting quantitation). Furthermore, since the sample and immunoreagents are applied

to the bottom of the wells of microtiter plates, effective washing between each immunoreaction to remove unbound material requires the use of special equipment to dispense and remove the wash buffer. Users of tests under field conditions (e.g., farms, homes, doctor's offices, and small clinics) would benefit from faster and simpler EIA. As compared to the non-porous solid phases, macroporous cloths of hydrophobic fibers provide a much larger surface area, can accommodate a larger volume of sample, and allow for easier washing between each immunoreaction. These attributes should enable cloth to provide rapid and simple EIA.

The present chapter demonstrates these advantages of hydrophobic cloth as an immunoadsorbent for EIA. As an example, this chapter will examine the use of bovine serum albumin (BSA) as a model protein antigen immobilized on the solid phase for the assay of anti-BSA immunoglobulin G (IgG) antibody in rabbit serum.

MATERIALS AND METHODS

Materials

The following reagents were obtained from Sigma Chemical Co.: bovine serum albumin (BSA) (No. A-7030), 2,2'-

ar'no-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (No. A-1888), rabbit anti-BSA serum (No. B-1520), normal rabbit serum (No. S-2632), and goat anti-rabbit IgG antibody-peroxidase conjugate solution (No. A-6154). The conjugate was diluted 1:1000 in 0.01 M phosphate buffered (pH 7.2)-0.85% NaCl (PBS) containing 0.05% Tween 20 (PBST) before use.

The following were examined as immunoadsorbents for EIA: polyester nonwoven cloth (DuPont, Sontara 8100), polypropylene cloth (Aldrich, No. Z10425-6), polyethylene filter (Fisher Scientific, 1.5 mm thickness), cellulose cotton cloth (obtained locally), and 96-well polystyrene microtiter plates (Bio-Rad, No. 224-0096).

Preparation of BSA-coated solid phase

Cloths were cut into 6 mm square segments, and each segment was incubated with 50 μ l of BSA solution (10 μ g/ml in PBS) for 16 h at room temperature. Each cloth was then washed with 5 ml of PBST on a vacuum filtration apparatus, and then stored in PBS at 4 °C until use. The antigenic activity of the cloths (as determined by CEIA-see below) remained unchanged for at least 3 months. The microtiter plate was coated by incubating each well with 100 μ l of BSA

solution as above. The wells were then washed 8 times by filling with PBST, inverting the plate and shaking to remove residual buffer. BSA-coated plates were used immediately in the EIA.

Enzyme immunoassay

For antibody capture, 50 μ l of diluted rabbit serum was added to either a BSA-coated cloth segment placed in a petri dish or a BSA-coated microtiter plate well and incubated for 0.5 to 30 min at room temperature. The cloth segments were then placed on an absorbent pad (e.g., a disposable diaper, or as an economical alternative for processing larger numbers of cloths, Dignity extraduty absorbent pads, Humanicare Int., Inc., No. 26950) and washed 5 times dropwise with a total volume of about 1 ml of PBST per segment. The wells of the microtiter plate were washed as previously. The bound antibody was then detected by incubating each cloth segment or microtiter plate well with 50 μ l of anti-rabbit IgG-peroxidase conjugate for 1 to 30 min at room temperature. The cloths and microtiter plate wells were then washed with PBST as above, and each was shaken in 250 μ l of ABTS substrate solution (10 mM ABTS and 0.5 mM H_2O_2 in 0.05 M sodium citrate buffer, pH 4.5) for 30

min at room temperature (incubations of the cloths with substrate were carried out in individual test tubes, and the developed substrate solution was then transferred to unused wells of the microtiter plate for absorbance measurements. The wells of the microtiter plate were incubated directly with substrate). The absorbance of the developed substrate solution at 414 nm (A_{414}) was then measured using an EIA plate reader (Bio-Tek Inc., Model No. EL 307).

RESULTS

Comparison of various cloths as immunoabsorbents for EIA

Four types of commercially available cloths were compared as immunoabsorbents for the cloth-based EIA (CEIA). The cloths were coated with BSA, and were then incubated for 30 min with either anti-BSA rabbit serum (diluted 1:100 000 in PBST) or similarly diluted normal rabbit serum devoid of anti-BSA antibodies (negative control). After washing the cloths with PBST to remove unbound serum components captured anti-BSA IgG was detected by incubation with the anti-rabbit IgG-peroxidase conjugate for 30 min, followed by the peroxidase assay as described in Methods.

Table 1.1 shows that the highest specific EIA signal (A_{414} value) was obtained with the BSA-coated polyester

cloth. All of the cloths, with the exception of cotton cloth, exhibited negligible background signals when normal rabbit serum was applied, thus confirming the specificity of this CEIA. Polyester cloth was used as a solid phase in subsequent experiments.

Table 1.1. Comparison of the EIA performed on various BSA-coated cloths.

Cloth	A_{414} ^a	
	anti-BSA serum	normal serum
polyester	0.38 ± 0.02	0.02 ± 0.0
polypropylene	0.12 ± 0.03	0.03 ± 0.0
polyethylene	0.30 ± 0.02	0.03 ± 0.0
cotton	0.25 ± 0.04	0.18 ± 0.03

^a mean A_{414} value $\pm$ standard error (n=4).

Comparison of cloth and the microtiter plate in EIA

The effect of the high surface area of the macroporous polyester cloth on its performance as an immunoadsorbent for EIA was examined by comparison to the non-porous microtiter plate in the assay of anti-BSA IgG over a wide range of antibody concentrations. A series of BSA-coated polyester cloth segments and microtiter plate wells were incubated for

30 min with doubling dilutions of the anti-BSA or normal serum (negative control) in PBST. After washing with PBST, the cloths and microtiter plate wells were incubated for 30 min with the anti-rabbit IgG-peroxidase conjugate, then washed again and assayed in peroxidase substrate as before. Fig. 1.1 shows that at the lower serum dilutions the polyester cloth produced significantly greater EIA signals than the microtiter plate. This difference decreased at the higher dilutions, and both immunoadsorbents gave a similar limit of detection (i.e., maximum serum dilution capable of producing a measurable EIA signal-about 1:256 000). The greater signals obtained at the lower serum dilutions suggest that BSA-coated polyester cloth has a higher capacity for antibody capture, probably due to the high surface area of the cloth which allowed for the immobilization of a greater quantity of antigen than the limited surface of the microtiter plate. Thus, at the lower serum dilutions the BSA-coated polyester cloth provided a better quantitative assay for anti-BSA IgG. When the concentration of antibodies became sub-saturating at the higher dilutions, the microtiter plate performed as well as the cloth. The negative controls (cloths or wells incubated with normal serum) exhibited negligible background signals

Fig. 1.1. EIA using polyester cloth and a microtiter plate. The EIA signals obtained using BSA-coated polyester cloth and a similarly coated microtiter plate were compared over a wide range of serum dilutions using 30 min incubations for both immunoreactions (i.e., antibody capture and detection with conjugate). Data are plotted as mean A_{414} value $\pm$ standard error (n=4). Symbols: ●, ■ polyester cloth; ○, □ microtiter plate; ●, ○ anti-BSA serum; ■, □ normal serum.

at all dilutions tested.

Comparison of the kinetics of immunoreaction

Polyester cloth (DuPont, Sontara 8100) has a thickness of ca. 1 mm and its 6 mm square segment totally absorbed the 50 μ l of sample and conjugate applied, permitting all of the molecules in solution to react over a large surface area. On the other hand, the microtiter plate well provided only a partial contact with the sample molecules over a limited surface area. Using this advantage of the cloth, it should be possible to reduce the length of time required to obtain measurable immunoreactions in the EIA. Here the kinetics of immunoreactions on both polyester cloth and the microtiter plate are examined.

BSA-coated cloth segments and microtiter plate wells were incubated with either anti-BSA or normal serum (diluted 1:100 000 in PBST) for 0.5-20 min, and then washed. They were then incubated for 30 min with the anti-rabbit IgG-peroxidase conjugate, then washed again and assayed in the peroxidase substrate. Fig. 1.2A shows that significant EIA signals (arbitrarily defined as $A_{414} > 0.2$) could be produced in as short as 0.5 min incubation of the anti-BSA serum with the cloth and only after 5 min incubation with the

Fig. 1.2. Effect of incubation times on the EIA. Part A shows the effect of varying the incubation time with serum from 0.5 to 20 min while keeping the conjugate incubation time fixed at 30 min. Part B shows the effect of keeping the serum incubation fixed at 2 min while varying the conjugate incubation from 1 to 20 min. Symbols: ●, ■ polyester cloth; ○, □ microtiter plate; ●, ○ anti-BSA serum; ■, □ normal serum. Data are plotted as mean A_{414} value $\pm$ standard error (n=4).

microtiter plate.

The time for the EIA can be further shortened by reducing the time of the second immunoreaction (i.e., with conjugate). To demonstrate this, the above comparison was repeated using a fixed incubation period of 2 min for the first immunoreaction (i.e., with anti-BSA serum) while varying the period of the second immunoreaction. Fig. 1.2B shows that a 10 min second immunoreaction was sufficient to produce a significant signal in the EIA on polyester cloth, whereas the microtiter plate produced only weak signals throughout the range of incubation periods tested.

DISCUSSION

The present results demonstrate that macroporous cloths of hydrophobic fibers, such as polyester cloth, have important advantages as immunoadsorbents for EIA. As compared to non-porous solid phases such as microtiter plates, they accommodate larger volumes of sample for immediate immunoreaction over a large surface, thus yielding faster immunoreactions. This in turn permits the development of rapid EIA. Also, the large surface area available for immunoreactant immobilization broadens the range of sample concentrations over which the assay remains

quantitative.

Another advantage of cloths is that they possess excellent filtration characteristics and can therefore be readily washed between each immunoreaction with small volumes of the wash buffer without the need for special equipment. The method used presently involves washing the cloth on an absorbent pad (e.g., disposable diaper) dropwise with a small volume of the buffer. The absorbent pad can be placed in a sealable disposable container if the test sample is either toxic or pathogenic and must be contained. Alternatively, the cloths can be effectively washed between immunoreactions using a vacuum filtration apparatus if containment is unnecessary.

A variety of other porous solid phases have previously been tested as immunoabsorbents for EIA, including microporous membranes of cellulose nitrate and cellulose acetate, and an analytical filter paper (Blais et al., 1989). However, the membranes could not be washed effectively between each immunoreaction using the absorbent pad. Even when washed with copious volumes of buffer on a vacuum filtration apparatus they still exhibited high non-specific EIA signals for negative controls, indicating that non-specific adsorption of the conjugate was a problem. The

filter paper similarly suffered high background signals. On the other hand, certain macroporous cloths such as polyester cloth have a low capacity for non-specific adsorption of conjugate, allowing for the production of high specific EIA signals while exhibiting negligible backgrounds. All of these advantages make polyester cloth an ideal immunoabsorbent for EIA, particularly for performing assays under field conditions where relatively small numbers (e.g., a few dozen) of samples need to be tested.

In the following chapters, these advantages of cloth will be further examined in the development of rapid, quantitative, and sensitive cloth-based EIA (CEIA) for the detection of specific antibodies and antigens.

CHAPTER 2

RAPID ANTIBODY ASSAY BASED ON THE INITIAL RATE

OF IMMUNOREACTION

ABSTRACT

A rapid cloth-based enzyme immunoassay (CEIA) for the assay of specific antibodies on the basis of the initial rate of immunoreaction was developed using a Salmonella antigen-antibody system. Salmonella typhimurium lipopolysaccharide-coated polyester cloth was instantaneously exposed to goat anti-Salmonella antibody standards, and the captured antibodies were detected using an anti-goat antibody-peroxidase conjugate. The enzyme signals produced in the CEIA were proportional to the antibody concentration, thus providing the basis for a rapid and quantitative assay of antibodies. Using this approach, the antibody response of a rabbit immunized with S. typhimurium antigens was followed over time, and the CEIA accurately measured the changes occurring in the anti-Salmonella antibody titer.

INTRODUCTION

The quantitative assay of specific antibodies in clinical samples not only enables the clinician to determine whether exposure to a given infectious agent has occurred, but may also provide an important clue as to the stage of the infection (Richter, 1982). The EIA of specific

antibodies as used in disease diagnoses is commonly performed by either of two methods using antigen immobilized on a microtiter plate. The first, the so-called titration (or 'endpoint') method, involves assaying serial dilutions of the test sample (e.g., serum) in order to determine the maximum dilution which produces an enzyme signal nearest an arbitrarily assigned endpoint. This maximum dilution factor is known as the 'titer', and is a function of the test sample's antibody activity (which is the net effect of the average affinity of the specific antibodies as well as their concentration). Thus, samples with higher antibody activities yield higher titers. The second method for assaying specific antibodies uses a fixed sample dilution and then measures the extent of immunoreaction with the immobilized antigen (which is also a function of the antibody activity). Both methods rely on the completion of each immunoreaction, which usually requires in excess of 30 min, and are therefore termed 'equilibrium' EIA.

It is theoretically possible to assay antibodies on the basis of the initial rate of immunoreaction during an instantaneous exposure of the sample to the antigen-coated solid phase. However, such a strategy is rarely used because short immunoreactions on non-porous solid phases

such as microtiter plates do not yield significant signals. In chapter 1, it was demonstrated that the use of polyester cloth as an adsorbent for a protein antigen permits the assay of specific antibodies using short immunoreactions (i.e., several minutes) due to the large surface area available and the suitability of cloth for quick washing to remove unreacted sample material. Using this cloth-based EIA (CEIA), it may be possible to obtain measurable amounts of antibodies bound during the initial stage of immunoreaction. Such an assay using non-equilibrium immunoreaction times would not only be rapid, but should also be quantitative on the basis of the kinetics of antibody capture, the initial rate of which would be proportional to the antibody concentration. The present chapter examines this possibility using the capture of anti-Salmonella antibodies by Salmonella typhimurium lipopolysaccharide (LPS)-coated polyester cloth. The Salmonella LPS molecule, a major cell surface antigen, contains a hydrophobic lipid A moiety which permits its immobilization on the polyester cloth by hydrophobic interaction. The structure of S. typhimurium LPS is shown in Fig. 2.1. It will be shown here that the initial rate of antibody binding to immobilized LPS antigens is indeed

Fig. 2.1. Structure of Salmonella typhimurium LPS. The basic structural unit of the LPS molecule consists of three regions, designated O-side chain (O-chain), core and lipid A. The first two regions consist of saccharides, and the third of a saccharide and a lipid. The lipid A region, which is highly hydrophobic, serves to anchor the molecule in the outer membrane of the bacterium. The composition and structure of the core region is common to all members of the genus Salmonella, while that of the O-chain varies among different strains (or serotypes). Sugars: heptose (Hep), glucose (Glu), galactose (Gal), N-acetylglucosamine (GlcNAc), rhamnose (Rha), mannose (Man), abequose (Abe), and keto-deoxyoctonate (KDO).

proportional to the level of antibody in the sample, thus permitting the rapid and quantitative assay of the antibody.

MATERIALS AND METHODS

Antibody standard and enzyme conjugates

The antibody standard used was a commercial preparation of affinity-purified polyclonal goat antibodies (CSA-1) to heat-killed Salmonella cells (Kirkegaard and Perry Laboratories, Inc., No. 01-91-99), with a specificity for the heat-stable antigens (predominantly lipopolysaccharides-see chapter 5) of all known Salmonella serotypes. A stock of the CSA-1 antibody (1 mg/ml in PBS) was stored at -20 °C. Other immunoreagents used were a rabbit anti-goat IgG antibody-peroxidase conjugate solution (Sigma, No. A-3540) and a goat anti-rabbit IgG antibody-peroxidase conjugate (Sigma, No. A-6154). Before use, these conjugate stock solutions were diluted 1:1000 in PBST.

Preparation of Salmonella antigens for immunization

Salmonella typhimurium strain LT2 (Salmonella Genetic Stock Center, University of Calgary) stored at 4 °C on a nutrient agar slant was inoculated into M63 minimal salts medium supplemented with 0.5% glucose (Miller, 1972) and

grown to a density of about 10^9 cells/ml (late log phase) by shaking at 37 °C for 16 h. The cells were washed twice with PBS by centrifugation at 10 000 X g for 10 min, 4 °C, and resuspended in PBS containing 50 mM ethylenediaminetetraacetate (EDTA) (pH 7.2) to a density of about 10^9 cells/ml. The suspension was then autoclaved at 121 °C for 20 min, cooled to room temperature, and dialysed extensively against PBS at 4 °C. Heating in the presence of EDTA not only kills the cells, but has also been shown to extract (i.e., solubilize) the LPS antigens from the cell surface (see chapter 5). This crude antigen suspension was stored at 4 °C.

Preparation of rabbit anti-Salmonella serum

For immunization, a 3 month old male New Zealand White rabbit was inoculated intravenously (in the marginal ear vein) with 0.5 ml of the crude antigen suspension (day 1). After 20 days, the rabbit received a booster injection (0.5 ml of the antigen suspension administered intravenously). Serum samples were prepared from bleedings done on days 1 and 20 (prior to the inoculations) and at 10-day intervals thereafter. Serum was prepared by incubating freshly collected blood at 37 °C for 2 h, allowing clot formation,

followed by centrifugation at 5 000 X g for 10 min, 4 °C, to remove clots and red blood cells. The sera were then stored at -20 °C until use.

Preparation of LPS-coated polyester cloth for the CEIA

S. typhimurium LPS (Sigma, No. L-6511) was suspended at 10 µg/ml in PBS containing 50 mM EDTA and then completely solubilized by heating at 100 °C for 10 min. Segments (6 mm squares) of polyester cloth (DuPont, Sontara 8100) were each incubated with 50 µl of the cooled LPS solution for 16 h at room temperature, then washed with PBST and stored at 4 °C in PBS. The antigen activity of the LPS-coated polyester cloth (LPS-cloth) remained stable for at least 3 months.

Determination of the initial rate of immunoreaction on LPS-cloth

The LPS-cloth segments were placed on an absorbent pad using forceps, and 100 µl of goat antibody standard or rabbit serum diluted in PBS was pipetted onto each segment, one at a time, and allowed to be drawn through the cloth by the absorbing action of the pad. The cloth segments were then immediately rinsed dropwise with ca. 0.5 ml of PBST to remove unreacted sample, and transferred to a petri dish.

Bound IgG was detected by incubating each segment with 50 μ l of the anti-goat (or anti-rabbit) IgG antibody-peroxidase conjugate for 2 to 10 min at room temperature. The segments were then washed with PBST as before, and then transferred to individual 16 X 100 mm test tubes and incubated with 0.5 ml of ABTS substrate solution for 30 min at room temperature. The enzyme reaction was stopped by the addition of 0.5 ml of 0.1 M NaF, and the absorbance at 414 nm (A_{414}) was determined.

'Equilibrium' EIA using a microtiter plate

A 96-well polystyrene microtiter plate (Corning, No. 25805-96) was incubated with 200 μ l/well of a 2 μ g/ml S. typhimurium LPS solution (prepared as above) for 16 h at room temperature. The wells were then washed 4 times with PBST, and then incubated with 100 μ l of rabbit serum diluted in PBS for 30 min at room temperature (for determination of antibody titers by the endpoint method, serial two-fold dilutions of the serum were used). The plate was then washed with PBST as above, and incubated with 100 μ l/well of the anti-rabbit IgG antibody-peroxidase conjugate for 30 min at room temperature. After washing with PBST, each well was then incubated with 200 μ l of ABTS substrate solution for 30

min at room temperature, and the A_{414} of the solution was measured using a microtiter plate reader as previously. Serum titers determined by the endpoint method are expressed as the maximum dilution factor which produced an EIA signal nearest an A_{414} value of 0.2.

RESULTS

Assay of goat anti-Salmonella antibody based on the initial rate of immunoreaction

The advantages of the CEIA in providing fast immunoreactions and ease of washing on an absorbent pad (chapter 1) were applied here to measure the initial rate of immunoreaction during an instantaneous exposure of the antibody to the immobilized antigen (first immunoreaction). Segments (6 mm squares) of polyester cloth were coated with Salmonella LPS and placed on a water absorbing pad. One hundred μ l of affinity-purified goat anti-Salmonella antibody at various concentrations was passively absorbed through the segments and into the pad, and the segments were then incubated for either 2, 5 or 10 min with anti-goat IgG antibody-peroxidase conjugate (second immunoreaction), then washed and developed in the peroxidase chromogenic substrate solution for 30 min. Fig. 2.2 shows that the resulting

Fig. 2.2. Measurement of anti-Salmonella antibody by the initial rate of immunoreaction. LPS-cloth segments were instantaneously reacted with a goat anti-Salmonella antibody standard at various concentrations, and then incubated with an anti-goat IgG antibody-peroxidase conjugate for either 2 (●), 5 (○) or 10 (Δ) min, then assayed for bound peroxidase activity as described in Methods. Bound enzyme activity is expressed as mean $A_{414} \pm$ standard error (n=4).

colour intensity (CEIA signal) was proportional to the concentration of the antibody standard within certain concentration ranges (the breadth of which decreased with increasing times of the second immunoreaction). Therefore, this method, termed 'instantaneous' CEIA, permitted the quantitative assay of the antibody on the basis of the initial rate of immunoreaction observed during the instantaneous exposure of antibody to the immobilized antigen. Longer second immunoreactions produced higher signals, but the increase was not linearly proportional to the reaction time since the amount of conjugate became rate-limiting with longer reactions. The use of more concentrated conjugate should increase the signals and the range of proportionality, if desired.

Assay of anti-Salmonella antibody in serum

Although the 'instantaneous' CEIA allowed for quantitation of the purified antibody, it was necessary to evaluate its usefulness in the quantitative assay of antibodies in an animal model. Therefore, the assay was used to monitor changes occurring in the levels of specific antibodies in a rabbit during progression of the immune response after inoculation with antigen. A rabbit was

intravenously injected with Salmonella antigens to induce the formation of serum antibodies to the LPS antigen. After 20 days from the first injection, a second injection of antigen was administered to allow for a rapid increase in antibodies (predominantly IgG). Serum sampled at different times was then assayed by both the 'instantaneous' CEIA and the 'equilibrium' EIA (using prolonged immunoreactions on an LPS-coated microtiter plate) as described in Methods. Since the effective quantitation of antibody occurs within a certain range of concentrations (Fig. 2.2), it was necessary to dilute the serum samples prior to assay in order to achieve a suitable sample concentration. Fig. 2.3A shows that when serum was diluted 1:100 the 'instantaneous' CEIA was able to detect definite changes in the anti-Salmonella antibody level over time in a manner reflected by the titer values obtained using the endpoint 'equilibrium' EIA method (see the figure legend). When fixed serum dilutions were incubated with the wells of an LPS-coated microtiter plate using 30 min first and second immunoreactions a similar pattern of antibody detection was obtained at a serum dilution of 1:1000 (Fig. 2.3B). Serum taken from the animal on day 1 (prior to immunization) was also tested at various dilutions and failed to produce any detectable signal in

Fig. 2.3. Assay of anti-Salmonella antibody in rabbit serum. A rabbit was given two intravenous injections of a S. typhimurium antigen suspension twenty days apart. Serum was sampled on day 1 (prior to first injection), day 20 (prior to second injection), and at ten-day intervals thereafter. Serum samples were diluted 1:10 (●), 1:100 (○), 1:1000 (▲), 1:10 000 (△), or 1:20 000 (■) in PBS, and were then assayed for anti-Salmonella antibody using (A) the 'instantaneous' CEIA (using a 5 min second immunoreaction) as described in Methods, or (B) an 'equilibrium' EIA using 30 min first and second immunoreactions with the wells of an LPS-coated microtiter plate as described in Methods. A pre-immunization rabbit serum sample failed to produce measurable enzyme signals at any dilution when tested by these two methods. Bound enzyme signals are expressed as mean $A_{414} \pm$ standard error (n=4). Anti-Salmonella IgG titers were determined for each serum sample using the endpoint 'equilibrium' EIA on an LPS-coated microtiter plate as described in Methods. Serum titers were: 0 (day 1), 3 260 (day 20) and 13 040 (days 30 and 40).

either assay, thus confirming the specificity of these assays. Thus, the 'instantaneous' CEIA was able to detect changes in the serum antibody level of an immunized animal during progression of the immune response in a manner comparable to the more conventional 'equilibrium' EIA methods.

DISCUSSION

These experiments demonstrate the use of antigen-coated polyester cloth in the rapid measurement of specific antibodies on the basis of the initial rate of immunoreaction of antibody with the immobilized antigen. This 'instantaneous' CEIA required only one third of the time necessary to complete the 'equilibrium' EIA using a microtiter plate. This time may be further reduced by the use of a more sensitive chromogenic enzyme substrate, such as 3,3',5,5'-tetramethylbenzidine (Hosoda *et al.*, 1986), which would permit the more rapid attainment of measurable enzyme signals. Furthermore, this initial rate method may eliminate problems with false positive reactions caused by non-specific interactions of test sample components with the solid phase, since only highly specific antibodies would be expected to bind during the instantaneous incubation with

the LPS-cloth. False positive reactions are commonly encountered in 'equilibrium' EIA, and are more likely to occur using prolonged incubation times (Tijssen, 1985). This rapid assay was made possible by the use of a macroporous cloth as the solid phase, since the cloth provides a large surface for rapid immunoreaction with the antibody and allows for immediate washing after sample application to give an initial rate determination.

The 'instantaneous' CEIA approach is particularly well suited for field assays, since it is rapid, simple to perform and does not require special equipment. In instances where a colourimeter is not available for measuring the colour intensity of the developed enzyme substrate solution, semi-quantitative test results can be obtained by visual comparison of the test sample to appropriate antibody standards. The method should be generally useful as an immunodiagnostic tool for screening smaller numbers (e.g., a few dozen) of samples for the presence of antibodies to specific pathogens, provided that the antigens of interest can be immobilized on polyester cloth. In a suggested modification of the present method, large sheets of antigen-coated polyester cloth able to accommodate multiple samples can be used in a dot blot

procedure for the qualitative assay of antibodies, where small volumes of test sera are spotted at regular intervals on the cloth, followed by washing and then saturating the cloth sheet with a suitable anti-globulin antibody-enzyme conjugate which binds to the antibody. The bound enzyme (e.g., peroxidase) can then be detected by using a chromogenic substrate system yielding an insoluble product (e.g., 4-chloro-1-naphthol, 3,3-diaminobenzidine; Hawkes et al., 1982). In such a system, samples containing antigen-specific antibodies manifest a localized coloured reaction (or spot) on the cloth upon incubation with the substrate, thereby allowing their visualization. Such a dot blot procedure should be extremely useful not only for assaying clinical samples, but also for the rapid screening of hybridoma cultures for the presence of specific monoclonal antibodies.

In the following chapter, a rapid CEIA procedure for the detection of anti-Salmonella antibodies in chicken egg yolk shall be described. This procedure, which is based on the principles of the rapid CEIA elaborated upon in the present and previous chapters, will be useful as an immunodiagnostic test for the monitoring of exposure to salmonellae in chickens and as a means of assessing sanitary

conditions in rearing facilities. It therefore represents an important application of the advantages of polyester cloth.

CHAPTER 3

**DETECTION OF ANTI-SALMONELLA IgG IN CHICKEN EGG
YOLKS BY A RAPID CLOTH-BASED ENZYME IMMUNOASSAY**

ABSTRACT

A simple and rapid method for assaying anti-Salmonella IgG in chicken egg yolk is described. Polyester cloth coated with Salmonella lipopolysaccharide was used to capture specific antibodies in egg yolk, which were then detected using an anti-chicken IgG-peroxidase conjugate. This assay, requiring less than 30 min to complete, revealed considerable differences in the relative levels of anti-Salmonella IgG in the yolks of eggs obtained from various sources. Anti-Salmonella IgG levels were generally low in eggs obtained from large produce retailers, but were frequently elevated in eggs obtained from a small local farm. This assay may provide a simple and economical means of monitoring the levels of Salmonella contamination in chicken rearing facilities.

INTRODUCTION

One of the major sources of salmonellosis in man is poultry products. Owing to the increasing demand for poultry, there is a great need for rapid and economical methods for monitoring Salmonella contamination in the poultry industry. Efforts have thus far focused largely on the detection of viable Salmonella cells or their antigens

in poultry meat by standard culture methods (Association of Official Analytical Chemists, 1984) or by a combination of enrichment culture methods and rapid assays (see General Introduction). However, these methods are laborious and time consuming, usually requiring a maximum of 7 or a minimum of 2 days, respectively, before test results can be obtained for individual samples. Furthermore, it is difficult to determine whether Salmonella contamination has occurred at the rearing facility or the poultry processing plant, since tests are usually carried out on processed meat. It would therefore be advantageous to have a rapid test for detecting exposure to Salmonella in the poultry rearing facility so that the primary source of contamination can be controlled.

The present chapter examines the possibility of monitoring the exposure of chickens to salmonellae by assaying chicken egg yolks for the presence of specific antibodies. Chicken egg yolks frequently contain specific antibodies following the exposure of hens to bacterial or viral antigens as a result of the general immune response in these animals (Aulisio and Shelokov, 1969). Furthermore, antibodies to Salmonella have been found in a high proportion of both egg yolks (Thain and Blandford, 1981) and

sera (Thain and Cullen, 1978) from infected chickens. Methods for the detection of Salmonella-specific antibodies in chicken serum for the immunodiagnosis of salmonellosis include agglutination (Thain and Blandford, 1981) and haemagglutination (Smith et al., 1972) tests, which are based on the ability of the antibodies to cause visible agglutination of whole bacterial cells or antigen-coated red blood cells, respectively. Although not routinely used as a means of immunodiagnosis, specific antibodies in egg yolk have been detected by an agglutination test (Thain and Blandford, 1981) and an indirect immunofluorescence test (Aulisio and Shelokov, 1969), in which the antibodies are reacted with an antigen-coated glass coverslip and then detected by fluorescence microscopy after incubation with a fluorescein-labelled anti-chicken IgG antibody. The use of egg yolk rather than serum for the detection of specific antibodies in the routine monitoring of flocks for exposure to Salmonella would be simpler, less time consuming and would eliminate trauma to the animals incurred by bleeding.

The use of a macroporous hydrophobic polyester cloth as an adsorbent of antigens for the rapid capture and detection of specific antibodies in serum by enzyme immunoassay has been described in chapters 1 and 2. The macroporous cloth

should also be ideal for the rapid assay of antibodies in viscous samples such as egg yolk, in which the rate of antibody diffusion is limited by the viscosity, since the cloth provides a capture surface which is in close contact with the entire volume of the sample, therefore minimizing the distance through which the antibody must diffuse in order to interact with the immobilized antigens. Here it shall be demonstrated that the cloth-based EIA (CEIA) can provide a rapid assay for anti-Salmonella IgG antibodies in chicken egg yolks, revealing considerable differences in antibody levels in a sample of eggs originating from the Ottawa Valley region of Eastern Ontario.

MATERIALS AND METHODS

Antigens and immunoreagents

The following were obtained from Sigma Chemical Co.: Salmonella typhimurium lipopolysaccharide (LPS) (No. L-6511), S. enteritidis LPS (No. L-6011), S. minnesota LPS (No. L-6261), S. typhosa LPS (No. L-6386), Escherichia coli strain K-235 LPS (No. L-2143), E. coli serotype O127:B8 LPS (No. L-3129), Pseudomonas aeruginosa LPS (No. L-9143), and rabbit anti-chicken IgG antibody-peroxidase conjugate solution (No. A-9046). The conjugate was diluted 1:1000 in PBST before

use.

Eggs

A total of 113 fresh eggs were tested for anti-Salmonella IgG. Of these, 71 were purchased from several independent Ottawa Valley produce retailers, 24 were purchased from two major Eastern Ontario supermarket chains, and 18 were obtained from a small (i.e., less than 40 hens) Ottawa Valley farm.

Cloth-based enzyme immunoassay (CEIA)

Nonwoven polyester cloth (DuPont, Sontara 8100) was cut into 6 mm square segments. For coating the cloth segments, Salmonella typhimurium LPS was dissolved at 10 µg/ml in PBS containing 0.05 M EDTA (pH 7.2) and then heated at 100 °C for 10 min. Each cloth segment was then incubated with 50 µl of the EDTA-heat-treated LPS solution for 16 h at room temperature, and then washed with a total of 5 ml of PBST on a filter under suction. The LPS-coated cloth segments (LPS-cloth) were stored in PBS at 4 °C.

Chicken egg yolk was diluted 1:5 in PBS, and a 10 µl sample was incubated with each LPS-cloth for 5 min at room temperature in a petri dish. The cloths were then placed on

an absorbent pad (disposable diaper) and each segment was washed 10 times dropwise with a total of about 1 ml of PBST. The cloths were then returned to a clean petri dish and incubated with 50 μ l the anti-chicken IgG-peroxidase conjugate for 5 min at room temperature, then washed with PBST as above. Peroxidase was assayed by shaking each cloth segment in 0.5 ml of ABTS substrate solution for 15 min at room temperature. The enzyme reaction was stopped by the addition of 0.5 ml of 0.1 M NaF, and the A_{414} of the developed substrate solution was determined.

Specificity of egg yolk antibodies adsorbed onto LPS-cloth

Ten polyester cloth segments (6 mm squares) were incubated with 1 ml of an S. typhimurium LPS solution (100 μ g/ml) prepared as above for 16 h at room temperature. The cloths were then washed with PBS and incubated with 1 ml of egg yolk diluted 1:5 in PBS for 1 h at room temperature, and then washed with PBS. They were then blotted, and the adsorbed antibodies were eluted by shaking the cloths in 1 ml of 0.1 M glycine-HCl (pH 2.2) for 5 min at room temperature. The liquid containing the eluted antibodies was then removed and neutralized by the addition of 0.2 volume of 1.0 M Tris-HCl (pH 8.0). The eluted antibodies

were assayed immediately by the CEIA on polyester cloth coated with various LPS antigens.

RESULTS

CEIA of specific IgG in egg yolk

Since hens in rearing facilities are frequently exposed to salmonellae (Stavric, 1987), it was surmised that at least a fraction of eggs purchased from local retailers might contain measurable amounts of anti-Salmonella IgG. To demonstrate the presence of specific IgG in egg yolk, the yolks of a number of chicken eggs collected from various local independent produce retailers were screened by the CEIA using cloth coated with Salmonella or various non-Salmonella LPS antigens. Polyester cloth segments were coated with LPS from either S. typhimurium, E. coli K-235, E. coli 0127:B8, or P. aeruginosa, and were then used for the capture of IgG from the yolks. Since the yolks were extremely viscous (even after dilution in PBS), slowing the diffusion of the antibodies to the LPS-cloth surface, a 5 min immunoreaction was used for antibody capture rather than the instantaneous exposure used for serum in chapter 2. The captured IgG was then detected with the anti-chicken IgG antibody-peroxidase conjugate. Of the yolks screened

initially, 2 (yolks A and B) produced considerable CEIA signals using the Salmonella LPS-cloth (Table 3.1). These egg yolks also produced varying CEIA signals using the non-

Table 3.1. CEIA of anti-LPS IgG in egg yolk ^a

LPS	A ₄₁₄ ^b	
	Yolk A	Yolk B
<u>S. typhimurium</u>	1.35 ± 0.11	0.45 ± 0.04
<u>E. coli</u> K-235	0.80 ± 0.06	0.51 ± 0.03
<u>E. coli</u> 0127:B8	0.37 ± 0.05	0.31 ± 0.05
<u>P. aeruginosa</u>	0.16 ± 0.01	0.25 ± 0.03

^a Polyester cloth segments were coated with LPS from either S. typhimurium or a variety of non-Salmonella Gram-negative bacteria. These LPS-cloths were then used in the CEIA of IgG from several chicken egg yolks as described in Methods. The results obtained with two of these yolks (A and B) are presented here.

^b Mean A₄₁₄ value ± standard error (n=4).

Salmonella LPS-cloths. This suggests that in addition to anti-Salmonella IgG, the egg yolks also contain IgG specific for various non-Salmonella LPS antigens. Alternatively, some of the anti-Salmonella IgG may have cross-reacted with epitopes on the non-Salmonella LPS.

Specificity of the CEIA

In order to confirm that the CEIA signals obtained on the Salmonella LPS-cloth were due to the presence of anti-Salmonella IgG, antibodies from yolk A or B were reacted with polyester cloth loaded with S. typhimurium LPS, and then eluted with pH 2.2 buffer and immediately neutralized as described in Methods. Exposure to acidic conditions disrupts antibody-antigen interactions (Tijssen, 1985), and therefore allows for the recovery of immunospecifically bound antibodies from the LPS-cloth. The eluted antibodies were then subjected to the CEIA using the Salmonella LPS-cloth or various non-Salmonella LPS-cloths for antibody capture as before. Table 3.2 shows that IgG eluted from S. typhimurium LPS-cloth gave significant CEIA signals with the Salmonella LPS-cloth but not with the non-Salmonella LPS-cloths. This confirms that the CEIA signals obtained using the Salmonella LPS-cloth (Table 3.1) were due to the

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Table 3.2. Specificity of egg yolk IgG after elution from S. typhimurium LPS-cloth ^a

LPS	A ₄₁₄ ^b	
	Yolk A	Yolk B
<u>S. typhimurium</u>	0.51 ± 0.04	0.20 ± 0.02
<u>E. coli</u> K-235	0.08 ± 0.0	0.05 ± 0.0
<u>E. coli</u> O127:B8	0.09 ± 0.01	0.05 ± 0.0
<u>P. aeruginosa</u>	0.06 ± 0.0	0.04 ± 0.0

^a Antibodies from either egg yolk A or B (see Table 3.1 footnote) adsorbed to S. typhimurium LPS-cloth were eluted with pH 2.2 buffer and then immediately neutralized as described in Methods. The eluted samples were then subjected to the CEIA using polyester cloth segments coated with LPS from S. typhimurium or various other non-Salmonella Gram-negative bacteria.

^b Mean A₄₁₄ value ± standard error (n=4).

presence of anti-Salmonella IgG in the yolks.

The specificity of yolk A IgG for the LPS of Salmonella species other than S. typhimurium was also tested using the CEIA. Polyester cloth segments were coated with LPS from three Salmonella species in addition to S. typhimurium and used in the assay of serial dilutions of yolk A. Fig. 3.1

shows that cloth segments coated with S. typhimurium LPS produced the highest signals at all dilutions, followed by those coated with S. typhosa, then S. enteritidis, and finally S. minnesota. Uncoated cloth (no LPS) did not produce a significant signal at any dilution, confirming that the CEIA signals are due to the LPS on the cloth. Since S. typhimurium is one of the most common Salmonella contaminants of chickens, it is likely that the yolk contains predominantly anti-S. typhimurium IgG which shows cross-reactivity with some epitopes of the other Salmonella species. Antibody reactivities to the antigens of Salmonella species which were not tested may also be present. Since the signals progressively decreased with increasing dilutions of the yolk, this CEIA appears to be quantitative and should be useful for the measurement of anti-Salmonella IgG levels in egg yolks.

Relative anti-Salmonella IgG levels in eggs from various sources

Since polyester cloth coated with S. typhimurium LPS appears to be suitable for use in the CEIA of anti-Salmonella IgG in egg yolk, a number of eggs obtained from various sources were screened in order to determine if any

Fig. 3.1. Detection of egg yolk A IgG to various Salmonella species. Polyester cloth segments were coated with LPS from either S. typhimurium (O), S. typhosa (●), S. minnesota (▲), S. enteritidis (△), or no LPS (□). The cloths were then used in the CEIA of various dilutions of egg yolk A (Table 3.1) in PBS. CEIA signals are plotted as mean A_{414} value $\pm$ standard error (n=4).

fluctuation in anti-Salmonella IgG levels could be observed. Egg yolks were screened by the CEIA using S. typhimurium LPS-cloth and a fixed dilution of the yolk in order to measure differences in the extent of immunoreaction (as judged by the CEIA signal) according to the level of specific IgG present. Table 3.3 shows that out of 113 eggs screened, the majority of those obtained from 3 out of 4 local independent produce retailers and 2 major supermarket chains produced lower CEIA signals (i.e., within an arbitrarily defined A_{414} range of 0-0.6). Signals from the egg yolks of the remaining independent produce retailer were also generally low, but at least 2 of the yolks gave high signals ($A_{414} > 1.0$). On the other hand, the majority of eggs obtained from a small local farm produced generally high signals ($A_{414} > 0.6$), with only 6 out of 18 eggs tested responding in the lower ranges.

DISCUSSION

The present chapter has demonstrated the application of the CEIA method to the rapid assay of anti-Salmonella IgG in chicken egg yolk. The method appears to be specific and sensitive, permitting the detection of anti-Salmonella IgG even at high dilutions of the egg yolk (Fig. 3.1).

Table 3.3. Relative anti-Salmonella IgG levels in eggs from various sources ^a

Source ^b	No. of eggs within absorbance range					
	0-0.2	0.2-0.4	0.4-0.6	0.6-0.8	0.8-1.0	>1.0
1 (16)	11	4	1	0	0	0
2 (28)	17	6	2	1	0	2
3 (19)	15	4	0	0	0	0
4 (8)	1	5	2	0	0	0
5 (18)	3	3	0	1	4	7
6 (12)	8	4	0	0	0	0
7 (12)	7	4	1	0	0	0

^a Fresh egg yolks from a variety of sources were diluted 1:5 in PBS and assayed for anti-Salmonella IgG by the CEIA using S. typhimurium LPS-cloth. Yolks were assayed in triplicate (n=3) and the mean A_{414} value was used to determine the absorbance range (in 0.2 absorbance unit increments) assigned to each yolk.

^b Sources No. 1, 2, 3, and 4 are separate independent produce retailers, source No. 5 is a small local farm, and sources No. 6 and 7 are major supermarket chains. The numbers in brackets indicate the total number of eggs tested from each source.

The sensitivity of the assay can be increased by simply using longer immunoreactions to maximize antibody capture. However, of more practical importance is the ability of the CEIA to detect the specific antibodies rapidly, which will be a significant advantage in its application as a screening tool. Although no epidemiological information was available, it is tempting to speculate that those sources of eggs exhibiting a significant proportion of yolks giving high CEIA signals (hence, elevated anti-Salmonella IgG levels) have had some contact with Salmonella organisms. This is especially likely in the case of the small local farm, where sanitary conditions may not meet the same standards as rearing facilities supplying the larger retailers. The method should become useful as a tool for monitoring sanitary conditions in rearing facilities when it is firmly established that an elevated level of anti-Salmonella IgG in eggs is related to the extent of Salmonella contamination. When the use of individual cloth segments is too cumbersome for processing larger numbers of eggs, a dot blot format involving large sheets of LPS-cloth onto which multiple samples can be applied simultaneously (as described in chapter 2) might be useful for qualitative tests.

The first three chapters of this thesis have described the use of polyester cloth as a solid phase for the passive immobilization of antigens such as proteins (chapter 1) or lipopolysaccharides (chapters 2 and 3) in the assay of specific antibodies. This passive immobilization was possible because of the relative hydrophobicity of these antigens, which were able to interact with the hydrophobic cloth surface. However, in instances where it is desirable to immobilize hydrophilic antigens for use in antibody capture the hydrophobic surface properties of polyester cloth are of limited value, and it is necessary to employ means other than passive adsorption to achieve antigen immobilization. The following chapter will examine one method which allows for the immobilization of a hydrophilic antigen on the polyester cloth surface.

CHAPTER 4

ACTIVATION OF POLYESTER CLOTH FOR THE IMMOBILIZATION

OF A POLYSACCHARIDE ANTIGEN

ABSTRACT

A polysaccharide antigen was immobilized on polyester cloth using cyanuric chloride as a coupling agent, yielding an antigen-coated solid phase suitable for enzyme immunoassay. The method should be generally useful for coating polyester cloth with amino or hydroxyl containing antigens or haptens which otherwise adsorb poorly to the cloth.

INTRODUCTION

The detection of specific antibodies in serum or other body fluids as used in the immunodiagnosis of infectious diseases by EIA requires the immobilization of antigen(s) on a solid phase for antibody capture. The immobilization of bovine serum albumin (a model protein antigen) and various lipopolysaccharide (LPS) antigens on polyester cloth for the EIA of specific antibodies has been demonstrated in the previous chapters. These antigens could be passively adsorbed to the hydrophobic cloth surface. However, some useful hydrophilic antigenic substances, such as polysaccharides, peptides or fragments of larger complex antigens, adsorb poorly to the cloth surface due to their insufficient hydrophobicity. The use of such hydrophilic

antigens in the assay of antibodies is sometimes necessary in order to achieve appropriate specificities and eliminate problems with false positive reactions due to undesirable cross-reactivities. Examples of hydrophilic antigens that have been used in EIA procedures for the detection of specific antibodies include (1) a polysaccharide antigen derived from Brucella abortus LPS, the use of which allows for the distinction between animals (cattle, sheep, etc.) that produce antibodies as a consequence of active infection and those that have simply been vaccinated (the distinction being made possible by the use of an epitope to which an immune response is only manifested during active infection, and not during passive exposure to Brucella antigens; Holman et al., 1985); and, (2) a synthetic peptide antigen which encompasses the major immunodominant epitope of protein gp41 from the envelope of the human immunodeficiency virus (HIV), the etiological agent of AIDS, whose use allows for the more sensitive and specific detection of anti-HIV antibodies in the immunodiagnosis of AIDS (Van Brunt, 1988).

Hydrophilic antigens are commonly immobilized on plastic surfaces (e.g., polystyrene) by using a bridging molecule (e.g., a protein such as BSA which can be passively adsorbed to the plastic surface) to which the antigen can be

conjugated by use of a bifunctional cross-linking agent such as glutaraldehyde or p-benzoquinone (Tijssen, 1985).

However, this approach requires that both the antigen and the bridging molecule possess either amino or sulfhydryl groups capable of reacting with the cross-linker (see General Introduction). Since some hydrophilic antigenic determinants such as certain polysaccharides and haptens may lack the functional groups necessary for reaction with these cross-linkers, the use of an alternative cross-linking agent is necessary for their immobilization. Also, the immobilization of hydrophilic antigens directly on the solid phase without the use of an adsorbed bridging molecule would be simpler and should yield a more stably bound antigen. This would require that the solid phase surface possess reactive functional groups for activation with a cross-linking agent. In the case of polyester cloth, the terminal hydroxyl groups of the polyester polymers may provide suitable sites at which a cross-linking agent can be attached to allow for the covalent immobilization of hydrophilic antigens. However, this requires the use of a highly efficient cross-linking agent, since hydroxyl groups generally do not give good reactions with the more commonly used cross-linkers.

Cyanuric chloride is a chloro-s-triazine reactive at alkaline pH with a variety of nucleophiles such as amino, sulfhydryl and hydroxyl groups (Zerlotti, 1967), and which forms stable linkages with various polysaccharides and peptides (Finlay et al., 1978). The proposed mechanism for the attachment of a molecule to the polyester cloth surface via cyanuric chloride is shown in Fig. 4.1. The present chapter will demonstrate the activation of polyester cloth with cyanuric chloride for the immobilization of a polysaccharide antigen. As an example, the immobilization of a polysaccharide antigen from Salmonella LPS will be examined.

MATERIALS AND METHODS

Materials

The following chemicals were purchased: from Sigma Chemical Co., bovine serum albumin (BSA) (No. A-7888), Salmonella typhimurium LPS (No. L-6511), and cyanuric chloride (No. C-5393); and from Kirkegaard and Perry Laboratories, Inc., peroxidase-labelled affinity-purified antibody to common Salmonella antigen (CSA-1) (No. 04-91-99), and 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase microwell substrate system (No. 50-76-00). Cyanuric

Fig. 4.1. Cross-linking of a hydroxyl or amino group-containing molecule to the polyester cloth surface via cyanuric chloride. Cyanuric chloride reacts first with the free hydroxyl groups on the polyester cloth surface, followed by its reaction with the free hydroxyl or amino groups of the molecule to be immobilized.

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chloride was recrystallized twice from chloroform. The peroxidase-CSA-1 conjugate was dissolved in PBS to make a 0.1 mg/ml stock and used diluted to 1:2000 in PBST.

Preparation of a polysaccharide antigen from Salmonella LPS

A model polysaccharide antigen used for immobilization on polyester cloth was prepared from S. typhimurium LPS by mild acid hydrolysis as follows (Hancock and Poxton, 1988). Four mg of LPS were dissolved in 2 ml of 1% (v/v) acetic acid and heated at 100 °C for 90 min. The solution was then centrifuged at 10 000 g for 10 min, and the supernatant was extracted with an equal volume of chloroform-methanol (2:1, v/v), and the organic phase discarded. The aqueous phase containing the polysaccharide was then dried by rotary evaporation, resuspended in distilled water and assayed for sugar content by the anthrone method (Hanson and Phillips, 1981) using S. typhimurium LPS as the standard.

Immobilization of a polysaccharide antigen

Non-woven polyester cloth (DuPont, Sontara 8100) was cut into 6-mm square segments which were then soaked for 30 min in 1 ml (per 10 segments) of a 5% (w/v) solution of cyanuric chloride dissolved in 1,4-dioxane. The cloths were

then transferred to a glass-scintered funnel and washed thoroughly with dioxane, followed by 0.1 M borate buffer (pH 9.2)(BB), then blotted. Each activated cloth was placed in a petri dish and immediately coated with 50 μ l of polysaccharide antigen diluted in BB, then incubated for 16 h at room temperature. Each segment was then washed with ca. 5 ml of PBST under vacuum. The unreacted cyanuric chloride residues were then blocked by incubating each segment with 50 μ l of 0.1 M ethanolamine buffer (pH 8) containing 1% (w/v) BSA, for 30 min at room temperature. They were then washed with PBST as above and stored in PBS at 4 °C until use.

EIA of bound polysaccharide antigen

Antigen-cloths were incubated with 50 μ l of the peroxidase-CSA-1 conjugate for 30 min at room temperature. The cloths were then washed with PBST as previously and incubated in 1.0 ml of TMB peroxidase microwell substrate system for 15 min at room temperature. Reactions were stopped by adding 0.1 ml of 0.5 M NaF and A_{370} was determined.

RESULTS

Effect of solvent

Since the availability of hydroxyl groups on the polyester cloth for reaction with cyanuric chloride may be influenced by the solvent, different organic solvents were examined. Cyanuric chloride was dissolved in either dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, or dioxane. Polyester cloth segments were then activated in cyanuric chloride in these solvents for 30 min and then coated with polysaccharide antigen from Salmonella LPS. The cloths were then blocked and assayed for bound antigen. Table 4.1 shows that cloths activated in either acetone or dioxane produced the highest EIA signals (A_{370} value), followed by DMF and DMSO. Subsequent experiments were performed using dioxane as the solvent for cyanuric chloride.

Activation time

Polyester cloth segments were activated with cyanuric chloride for various times, then washed and incubated with the polysaccharide antigen for 16 h, followed by blocking solution. The bound antigen was then assayed using the peroxidase-CSA-1 conjugate as above. Activation of the

Table 4.1. Effect of solvent on activation with cyanuric chloride ^a

Solvent	Polysaccharide Antigen	A_{370} ^b
	($\mu\text{g/ml}$)	
DMF	0	0.05 ± 0.0
	50	0.62 ± 0.05
DMSO	0	0.03 ± 0.0
	50	0.52 ± 0.04
Acetone	0	0.06 ± 0.0
	50	0.75 ± 0.11
Dioxane	0	0.05 ± 0.0
	50	0.71 ± 0.05

^a Polyester cloth segments were incubated for 30 min in solutions of cyanuric chloride (5%) in DMF, DMSO, acetone, or dioxane. The cloths were then incubated with BB buffer containing 0 or 50 $\mu\text{g/ml}$ of polysaccharide antigen and assayed for bound antigen as described in Methods.

^b Mean A_{370} value $\pm$ standard error (n=4).

cloth was complete after 30 min incubation with cyanuric chloride (Fig. 4.2). Unactivated cloth exhibited a small capacity for adsorption of the antigen.

Effect of antigen concentration

Unactivated or cyanuric chloride-activated polyester cloth segments were coated with 0 to 100 μg of polysaccharide antigen per ml. The cloths were then blocked and the bound antigen was determined as above. Cyanuric chloride-activated polyester cloth produced significant EIA signals at all antigen concentrations tested (Fig. 4.3), with the maximum signal occurring at 50 $\mu\text{g}/\text{ml}$. Fig. 4.3 also shows that at lower concentrations (<50 $\mu\text{g}/\text{ml}$) the antigen failed to adsorb significantly to the unactivated cloths. The antigen adsorbed to the unactivated cloths at the higher concentrations was completely removed by heating the cloths in carbon tetrachloride for 3 h at 85 °C. The same treatment applied to similarly coated activated cloths reduced the bound enzyme signal by only ca. 15%. Therefore, the cyanuric chloride treatment allowed for the stable immobilization of the polysaccharide, probably by covalent linkage.

Fig. 4.2. Time course of polyester cloth activation with cyanuric chloride. Polyester cloth segments were activated in a solution of cyanuric chloride for various periods of time, then incubated with 50 (O) or 0 (●) µg/ml of polysaccharide antigen and assayed for bound antigen. Enzyme signals are expressed as mean A_{370} value $\pm$ standard error (n=4).

Fig. 4.3. Effect of polysaccharide antigen concentration on immobilization. Polyester cloth segments were activated with cyanuric chloride (O) or left unactivated (●). The cloths were then coated with various concentrations of polysaccharide antigen and assayed as described in Methods. Enzyme signals are expressed as mean A_{370} value $\pm$ standard error (n=4).

DISCUSSION

These experiments demonstrate that polyester cloth can be activated with cyanuric chloride to allow for the immobilization of a polysaccharide antigen from Salmonella LPS which otherwise adsorbs only poorly to the cloth. This method should facilitate the preparation of antigen-coated cloth in instances where unadsorbable antigens such as polysaccharides or peptides are desirable for EIA. When very small antigens (e.g., small oligopeptides) must be immobilized it may be necessary to first attach a spacer molecule to the antigen or the cloth surface in order to eliminate steric hinderance of the antigen-antibody interaction which may occur if the epitope lies too close to the cloth surface. The ability to activate polyester cloth for the immobilization of hydrophilic antigens should broaden the applications of the CEIA method as an immunodiagnostic tool.

Chapters 1 through 4 of the present thesis have focused largely on the use of antigen-coated polyester cloth for the detection of specific antibodies. However, it must be emphasized that the advantages of macroporous cloth as a solid phase for EIA are not limited to this particular application. The rapid immunoreactions, ease of handling

and excellent filtration characteristics all represent important aspects of the cloth which can avail themselves in many other useful applications. The remaining chapters of this thesis will demonstrate some of the important applications of these advantages of polyester cloth in the detection of Salmonella antigens.

CHAPTER 5

EXTRACTION OF SALMONELLA ANTIGENS IN NON-SEDIMENTABLE FORMS FOR ENZYME IMMUNOASSAY

ABSTRACT

Heating Salmonella typhimurium cells in ethylenediaminetetraacetate was found to dissociate its antigens into forms that are non-sedimentable at 10 000 X g. The treatment caused a marked increase in the rate of immunoreaction and the sensitivity in a cloth-based enzyme immunoassay for the detection of Salmonella antigens. The method permits the extraction of Salmonella antigens from solid-rich samples and the preparation of solid-free samples by means of centrifugation. When the level of the antigens in the supernatant was too low for the immunoassay, the antigens were readily concentrated by passing a large volume of the supernatant through a macroporous hydrophobic cloth coated with anti-Salmonella antibodies. Using this method, the detection of as few as 10 Salmonella cells per gram of chicken meat was possible within a total of 18 hours, which included 16 hours of growth in either tetrathionate, selenite cystine, or nutrient broths.

INTRODUCTION

Members of the genus Salmonella, the major cause of human enteritis worldwide, are often found associated with solid or semi-solid matter (e.g., food, feces, etc.) from

which they are disseminated to human hosts. The identification of salmonellae in foods or feces from infected humans and animals is an important first step in controlling the spread of the organism. The most recent methodology developed for the rapid detection of Salmonella antigens in foods such as poultry and other meats involves (1) the enrichment of the salmonellae after inoculation of food samples into liquid cultures, followed by (2) the detection of Salmonella antigens in enrichment cultures by EIA (Mattingly et al., 1985; Ibrahim and Lyons, 1987). Such EIA frequently employ the 'sandwich' format, where specific antibodies are immobilized on a solid phase for the capture of the antigen which is then detected by an antibody-enzyme conjugate (Fig. 1A). In some instances, sufficient numbers of salmonellae may be present in test samples to allow for their direct detection without the need for prior enrichment. This is true of samples such as feces from acutely infected individuals, who may shed large numbers of cells, and also may occasionally occur in food samples such as heavily contaminated or spoiled poultry meat.

The EIA of Salmonella antigens in solid-rich samples such as foods or feces requires that the antigens be extracted from the sample into a solid-free liquid in order

to allow for their free interaction with the immobilized immunoreagents. The dissociation of Salmonella antigens present in solid or semi-solid samples into non-sedimentable forms in a liquid would allow for the removal of sample solids by centrifugation, leaving a solid-free liquid containing the antigens. Furthermore, because of their smaller sizes the dissociated antigens obtained in the supernatant should exhibit faster immunoreactions with antibodies in the EIA than antigens associated with intact cells, since the smaller antigens will diffuse more rapidly to the solid phase surface.

Another way to accelerate immunoreactions in the EIA would be the use of a large-surfaced adsorbent for immunoreactants. The previous chapters have demonstrated that macroporous hydrophobic cloths such as polyester cloth can provide a much greater surface area for immunoreactions (thus, faster reaction rates) than non-porous surfaces such as microtiter plates. Furthermore, macroporous cloths exhibit excellent filtration characteristics, which in combination with the capacity for rapid immunoreactions should allow for the use of antibody-coated cloths for the capture of antigens from large volumes of sample by filtration, thus increasing the effective sensitivity of the

EIA by permitting the concentration of dilute antigens on the cloth surface. These advantages of macroporous cloth when combined with a method for the preparation of solid-free antigen samples should allow for a more rapid, sensitive and simple EIA for Salmonella antigens in solid-rich samples.

It will be shown here that Salmonella lipopolysaccharide (LPS) (the immunodominant cell surface antigen) can be effectively extracted from an inoculated solid food sample into a non-sedimentable form by heating the sample in the presence of the chelating agent ethylenediamine-tetraacetate (EDTA); that the extracted antigens react much faster with antibodies adsorbed onto the cloth; and that the antibody-coated cloth can be used to concentrate dilute antigens for detection by the cloth-based EIA (CEIA).

MATERIALS AND METHODS

Bacteria

Salmonella typhimurium strain LT2 was grown in M63 minimal salts medium (Miller, 1972) containing 0.5% glucose by shaking at 37 °C for 16 h. For use in the EIA, the Salmonella cells were washed twice with PBS by

centrifugation at 10 000 X g for 10 min, 4 °C, and resuspended in the original volume of PBS, giving a final density of 6×10^8 cells/ml (confirmed from viable counts obtained by plating serial dilutions of the washed cell suspension on nutrient agar and incubating the plates at 37 °C for 20 h). The cell suspension was used in the EIA within a few hours of its preparation.

Immunoreagents

The primary (capture) antibody used to coat the polyester cloth for EIA was a commercial preparation of affinity-purified polyclonal antibody (CSA-1) to heat-killed Salmonella cells (Kirkegaard and Perry Laboratories, Inc., No. 01-91-99) with a specificity for all known Salmonella serotypes. The immunological specificity of a CSA-1 antibody-horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Inc., No. 04-91-99) has been tested by CEIA, and approximately 60% of the total enzyme activity bound to purified S. typhimurium LPS (Sigma, No. L-6511) loaded onto polyester cloth. A stock of the conjugate (0.1 mg/ml in PBS) was stored at -20 °C. Before use, it was diluted 1:2000 in PBST.

Cloth-based enzyme immunoassay (CEIA)

The cloth-enzyme immunoassay (CEIA) using polyester cloth as an antibody adsorbent for the capture of antigens from test samples was performed as follows. Polyester cloth (DuPont, Sontara 8100) was cut into 6 mm square segments. Each segment was incubated with 50 μ l of the CSA-1 antibody (50 μ g/ml in PBS) for 6-16 h at room temperature, then thoroughly washed with 5 ml PBST using a vacuum filtration apparatus. The antibody-coated cloths were stored in PBS at 4 °C and remained stable for at least 3 months. Fifty μ l of antigen sample was pipetted onto each antibody-coated cloth in a petri dish and incubated for 30 min (unless otherwise stated) at room temperature, and then the cloths were placed on an absorbent pad (disposable diaper) and washed 5 times dropwise with a total of about 0.3 ml of PBST. The cloths were returned to a clean petri dish and incubated with 50 μ l of the CSA-1 antibody-horseradish peroxidase conjugate for 30 min at room temperature. The cloths were then washed with PBST as before and bound enzyme was assayed by incubating each segment in 0.5 ml of ABTS substrate solution (Chapter 1) for 30 min at room temperature. The reaction was stopped by the addition of 0.5 ml of 0.1M NaF, and the absorbance at 414 nm (A_{414}) was determined.

Chicken breast paste

Fresh grade A chicken breast meat was obtained locally and blended to a paste using a food processor and then inoculated with washed Salmonella cells, then analyzed by the CEIA directly or after enrichment.

CEIA after concentration of antigens onto antibody-coated cloth

Polyester cloth was cut into 1 cm diameter discs and each was incubated with 100 µl of the CSA-1 antibody (50 µg/ml in PBS) for 6-16 h, at room temperature. Each disc was then inserted at the bottom of a 1 cm diameter QUIK-SEP disposable polypropylene column (Isolab, Inc. No. QS-U). Liquid samples containing antigen were then passed (by gravity flow) through the columns at a flow rate of 25-50 ml/h. Upon passage of the entire sample volumes through the columns, the discs were then incubated in situ (in the column) with 100 µl of the CSA-1 antibody-horseradish peroxidase conjugate for 30 min, washed with PBST (4 ml per column), and incubated in situ with 1 ml of ABTS substrate solution for 30 min with gentle agitation of the column. Reactions were stopped by the addition of 1 ml of 0.1 M NaF, and the absorbance (414 nm) of the effluent from the column

was determined.

RESULTS

Effect of EDTA and heat on CEIA of Salmonella antigen

Previously, treatment with 10 mM EDTA at room temperature has been shown to release LPS from the surface of Escherichia coli, Salmonella anatum, and S. minnesota (Leive and Shovlin, 1968). Also, heat-killed Salmonella cells have been widely used as a source of heat-stable antigens for antibody production (Ewing, 1986). Here the effect of both EDTA and heat treatments on the detection of Salmonella antigens by CEIA was studied using S. typhimurium as a model antigen. Washed S. typhimurium cells were suspended at 3×10^7 cells/ml in various concentrations of EDTA in PBS (EDTA-PBS, pH 7.2), and 1 ml portions were either heated at 100 °C for 10 min or left at room temperature. Fifty microliter suspensions (containing 1.5×10^6 cells) or EDTA-PBS alone (negative control) were then incubated for 30 min with 6 mm squares of polyester cloths which had been pre-coated with anti-Salmonella antibody. The captured antigen was detected by the antibody-horseradish peroxidase conjugate. Fig. 5.1 shows that EDTA treatments at room temperature enhanced the CEIA signal (at

Fig. 5.1. Effect of heating Salmonella cells in various concentrations of EDTA on the CEIA signal. Washed Salmonella cells were suspended at 3×10^7 cells/ml in PBS containing various concentrations of EDTA and heated at 100 °C for 10 min (●) or left at room temperature (O). These samples were then processed in the CEIA as described in Methods. A series of negative controls (no antigens) consisting of PBS containing various concentrations of EDTA alone (■) are also shown. The CEIA signals (A_{414}) are plotted as mean value $\pm$ standard error (n=3).

all EDTA concentrations tested) in comparison with the untreated cells. However, a combination of EDTA and heat treatment gave even greater enhancement. The EDTA enhancement was maximal at 50 mM EDTA and declined at the higher EDTA concentrations. Heating Salmonella cells in 50 mM EDTA caused a four-fold increase in the CEIA signal as compared to the untreated cells. The negative controls show negligible signals which were not affected by the EDTA treatment. At 50 mM EDTA, 2 min heating (of 1 ml of cell suspension) was found to be sufficient, but heating for at least 10 min is recommended to ensure the killing of all Salmonella cells for safer handling.

When EDTA (50 mM)-heat-treated cells were centrifuged at 10 000 X g for 10 min, approximately 85% of the total antigenic activity (determined by the CEIA) was found in the supernatant. This suggests that the treatment caused the dissociation of the cell-associated antigens into smaller units.

Kinetics of immunoreaction of the EDTA-heat-treated antigens

In the above studies, the EDTA-heat-treated cells were allowed 30 min to react with antibody adsorbed onto the cloth. The captured antigens were then detected by 30 min

incubation with the conjugate followed by 30 min incubation with the peroxidase substrate. Fig. 5.2 shows the effect of varying the immunoreaction time of EDTA (50 mM)-heat-treated and untreated cells with the adsorbed antibody, while maintaining other reaction conditions as above. After as little as 0.5 min immunoreaction, the treated cells gave significant CEIA signals, whereas the untreated cells produced only barely detectable signals. These results confirm that the EDTA-heat treatment dissociates Salmonella antigens, probably largely LPS, into smaller units which react faster with the adsorbed antibody.

Detection of Salmonella in chicken meat

Since EDTA-heat treatment allows for the extraction of Salmonella antigens into a supernatant after centrifugation, it should also be possible to prepare solid-free antigens from solid-rich Salmonella samples. As an example, the extraction of Salmonella antigens from chicken breast was examined. For this, concentrations of EDTA higher than 50 mM may be required since the effective concentration of EDTA will be reduced by the divalent ions present in the meat. Therefore, chicken breast paste was inoculated with Salmonella (6×10^8 cells per g of paste), and heated in

Fig. 5.2. Kinetics of the antibody-antigen reaction of EDTA-heat-treated and untreated Salmonella samples. Washed Salmonella cells were suspended at 3×10^7 cells/ ml in PBS with or without 50 mM EDTA. The suspension in EDTA-PBS was heated at 100 °C for 10 min (●) while the suspension in PBS (untreated sample) was left at room temperature (O). The samples were then incubated with antibody-coated cloths for various lengths of time then processed in the CEIA as described in Methods. The CEIA signals (A_{414}) are plotted as mean value $\pm$ standard error (n=3)

various EDTA concentrations. The antigens extracted in the supernatants after centrifugation as before were assayed by the CEIA. Fig. 5.3 shows that the EDTA-heat treatment extracted the antigens from the meat most efficiently in the range of 0.2 M to 0.5 M EDTA (judged from the CEIA signals) and that the uninoculated samples gave negligible signals (above the background) which were unaffected by the different EDTA concentrations.

Concentration of dissociated antigens on antibody-coated cloths

The above experiments show that Salmonella antigens can be extracted into a solid-free liquid. However, in practice the concentration of the antigens in the sample liquid may not be sufficiently high to be detected by the CEIA. Since larger volumes of the sample liquid can be passed through the antibody-coated cloth because of its macroporosity, it should be possible to concentrate the antigens on the cloth to a detectable level. To demonstrate this, 10 g samples of the chicken meat were inoculated with 1 ml of PBS containing various numbers of washed Salmonella cells. Solid-free antigen samples were then prepared by the addition of 50 ml of EDTA (0.2 M)-PBS to the inoculated meat and autoclaving

Fig. 5.3. Extraction of Salmonella antigens from chicken meat by EDTA-heat treatment. Samples (0.1 g) of chicken breast paste were mixed with 0.1 ml of PBS containing 6×10^7 Salmonella cells (●), or PBS alone (○), then heated at 100°C for 10 min in the presence of 0.5 ml of PBS containing various concentrations of EDTA. After cooling, the mixtures were centrifuged and the antigens in the supernatants were assayed by the CEIA (30 min immunoreactions with the antigens) as described in Methods. The CEIA signals (A_{414}) are plotted as mean value $\pm$ standard error (n=3).

at 121 °C for 5 min (this method gives more uniform heating of larger sample volumes, yet produces the same result as the previous protocol). The EDTA-heat-treated samples were then centrifuged and the supernatants were passed through the antibody-coated cloths. The concentrated antigens were assayed in situ (i.e., in the columns) by the CEIA. Fig. 5.4 shows that the EDTA-heat-extracted samples could be readily concentrated by filtration through the antibody-coated polyester cloth. After concentration, the CEIA detected Salmonella in the meat inoculated at a density of 2×10^3 cells/g, whereas only the densities higher than 2×10^5 cells/g could be detected without concentration. The CEIA signals increased with increasing levels of inoculation. It is expected that even lower cell densities could be detected by this method by filtering larger volumes of liquid sample, as the macroporous cloth does not encounter difficulties with clogging upon passage of colloidal samples, as do microporous filters such as nitrocellulose and nylon membranes.

Detection of Salmonella cells by combined enrichment-CEIA

The presence of extremely low numbers of viable Salmonella cells in food samples has traditionally required

Fig. 5.4. Concentration of dissociated Salmonella antigens onto antibody-coated cloth. Several 10 g samples of chicken breast paste were inoculated with various numbers of Salmonella cells and then autoclaved at 121 °C for 5 min in the presence of 50 ml of PBS containing 0.2 M EDTA (pH 7.2). Sample solids were then removed by centrifugation and the resulting supernatants were passed through antibody-coated cloth discs inserted at the bottom of columns. The captured antigens were then assayed by the CEIA after (●) or before (○) concentration on antibody-coated cloth, as described in Methods.

that the samples be enriched to higher cell densities by incubation in various growth media before identification. A series of pre-enrichment and selective enrichment steps which require a minimum of 2 days have been used to identify Salmonella by EIA on non-porous solid phases (Rigby, 1984; and, Ibrahim and Lyons, 1987). To demonstrate the possibility of detecting very low numbers of Salmonella cells by a combined enrichment-CEIA method, 5 g samples of the chicken meat were inoculated with 1 ml of PBS containing 50 Salmonella cells, and were then shaken for 16 h at 37 °C with 10 ml of either one of two commonly used selective broths, Difco tetrathionate and selenite cystine (Ibrahim and Lyons, 1987), or the non-selective Difco nutrient broth. Solid-free antigen samples were then prepared by EDTA-heat extraction, concentrated on the antibody-coated cloths, and assayed by the CEIA. Table 5.1 shows that all three broths tested easily allowed for the detection of the cells by the CEIA after the 16 h enrichment period, and all of these broths produced similar results in terms of the CEIA signals obtained. The reason for the elevated background signals observed in the uninoculated control samples is not clear, but the very large differences between the signals obtained for the inoculated samples and the controls allow for a very

Table 5.1. Combined enrichment-CEIA for the detection of Salmonella cells in chicken meat ^a

Enrichment broth	No. of cells/g meat	A ₄₁₄ ^b
tetrathionate	0	0.30 ± 0.03
	10	1.16 ± 0.08
selenite cystine	0	0.25 ± 0.03
	10	1.28 ± 0.06
nutrient broth	0	0.30 ± 0.02
	10	1.33 ± 0.06

^a Five g of the chicken breast paste was mixed with 1 ml of M 63 medium containing 0 or 50 Salmonella cells, then with 10 ml of enrichment broth, and incubated for 16 h at 37°C without shaking. The mixtures were then mixed with 2 ml of 1 M EDTA (pH 7.2) in PBS, autoclaved for 5 min, then centrifuged at 10 000 g for 10 min at 4 °C. The antigens in the supernatants were concentrated onto antibody-coated cloth and assayed by the CEIA as described in Methods.

^b mean A₄₁₄ value ± standard error (n=3).

clear distinction between the two. Although nutrient broth seems adequate for the present purpose, in practice the use of a selective broth is preferred in order to avoid competition by other contaminating microorganisms during enrichment. However, the sole use of a selective broth for enrichment may not permit the growth of damaged salmonellae present in nutrient-deficient samples. Therefore, initiating cultures in a non-selective broth followed by a transfer to selective conditions may prove to be the best compromise.

DISCUSSION

This chapter has demonstrated that heating Salmonella cells in EDTA leads to the dissociation of their antigens into non-sedimentable forms, which permits the preparation of solid-free liquid samples from solid-rich samples such as poultry meat for antigen detection by the CEIA. Furthermore, because of their smaller sizes, the dissociated antigens interact more efficiently with the antibody adsorbed onto the cloth than cell-associated antigens. It is also possible that dissociation causes the exposure of additional epitopes able to react with the antibody used in the present studies. The serotype-specific antigenic

determinants of the Salmonella LPS molecule (the immunodominant antigen of the cell surface) reside within the O-chain region, whereas the core oligosaccharide region contains epitopes that are common to all members of the genus (Hitchcock et al., 1986). Since the CSA-1 antibodies recognize a common (heat-stable) Salmonella antigen, their major target is probably one or more epitopes located within the core region, which may require the EDTA-heat treatment for optimal exposure. EDTA probably acts by helping to solubilize LPS molecules present in the outer membrane, which is normally stabilized by divalent cations such as magnesium and calcium (Hannecart-Pokorni et al., 1973). When these cations are chelated by EDTA, the outer membrane is destabilized, and soluble LPS is shed from the cell surface (Leive and Shovlin, 1968). The application of heat completes the solubilization process.

The possibility of detecting dilute EDTA-heat-treated antigens present in large volumes of liquid sample by concentration onto the antibody-coated cloth has also been demonstrated. The EDTA-heat extraction method, in combination with the ability to concentrate antigens on antibody-coated cloth, should enable the direct detection of moderate numbers (i.e., a few thousand cells) of salmonellae

present in bulky solid-rich samples such as spoiled meats. This has not previously been possible with conventional EIA using untreated samples, since the extraction of all (or most) of the antigens present in bulky solid-rich samples followed by their concentration on an antibody-coated solid phase is necessary to ensure detection when only moderate levels of contamination are present. When smaller numbers (i.e., < 1000 cells) of salmonellae are present, samples can be inoculated in a suitable enrichment broth to allow for multiplication of the cells prior to performing the CEIA. In solid-rich samples containing very high numbers of enterobacteria other than salmonellae, such as feces, potential problems with cross-reactions in the CEIA might be eliminated by the use of a monoclonal antibody specific for unique Salmonella epitopes instead of the polyclonal reagent used presently. The EDTA-heat extraction procedure should be applicable to the detection of other Gram-negative bacteria in solid-rich samples. Preliminary studies indicate its applicability to the detection of enteropathogenic Campylobacters.

This chapter has focused on an EIA procedure which is based on the use of specific antibodies adsorbed onto polyester cloth for the capture of LPS antigens from test

samples. Although the use of antibodies provides an excellent means of capturing specific antigens, other biomolecules displaying high affinities for certain types of antigens may serve the same function. The following chapter examines the possibility of employing polymyxin B sulfate, a polypeptide antibiotic which displays a high affinity for the LPS of most Gram-negative bacteria, as an alternative capture agent for use in the CEIA of LPS antigens.

CHAPTER 6

**USE OF POLYMYXIN-COATED POLYESTER CLOTH IN THE ENZYME
IMMUNOASSAY OF SALMONELLA LIPOPOLYSACCHARIDE ANTIGENS**

ABSTRACT

Polyester cloth coated with polymyxin B was used to capture Salmonella typhimurium lipopolysaccharide antigens which were then quantitatively or qualitatively assayed using a specific antibody-peroxidase conjugate. This simple, rapid method can be used to assay a large number of samples by employing a large sheet of the polymyxin-coated cloth onto which multiple samples can be blotted. The method is reproducible and economical, since polymyxin B is relatively inexpensive, stable and available in pure form.

INTRODUCTION

The advantages of using polyester cloth as an adsorbent of antigens or antibodies for EIA have been reported in the previous chapters. Among these advantages are the rapid immunoreactions obtained due to the large surface area of the cloth, and the ease of filtration and washing due to its macroporosity. In chapter 5, anti-Salmonella antibody-coated polyester cloth was used to capture Salmonella antigens which were then detected by an antibody-enzyme conjugate. The use of specific antibodies immobilized on a solid phase for the capture of antigens from test samples has long been the standard practice in EIA utilizing the

'sandwich' format (Yolken, 1982). However, antibodies (whether polyclonal or monoclonal) are relatively expensive reagents and are unstable, requiring cold storage of the antibody-coated cloth (antibody-cloth). Furthermore, the quality of most antibody preparations is subject to variations in both degree of purity and overall affinity from one batch to another. It will be shown here that polyester cloth coated with the antibiotic polymyxin B (polymyxin-cloth), which has an affinity for the LPS of Gram-negative bacteria, can be used to capture Salmonella LPS antigens for subsequent detection using a specific antibody-enzyme conjugate. This polymyxin-based CEIA provides an improvement in the detection of Gram-negative food pathogens.

Polymyxin B, which is produced by Bacillus polymyxa, is an antibiotic which is bactericidal to Gram-negative bacteria. The antibiotic is available in a pure form, is much less expensive than antibodies, and is stable between pH 2 and 7, even when boiled. Because of its affinity for bacterial LPS, polymyxin B covalently bound to cross-linked agarose has been employed for the removal of LPS pyrogens from solutions (Issekutz, 1983). Polymyxin B is a cyclic peptide with a short peptide side chain acylated at its N-

terminus with an eight- or nine-carbon fatty acid. The structure of polymyxin B is shown below. Polyester contains

R = 6-methyloctanoyl or 6-methylheptanoyl

DAB = diaminobutyric acid

hydrophobic terephthalate residues. Therefore, it is expected that polymyxin B can be adsorbed hydrophobically to polyester cloth via its fatty acid moiety, leaving its peptide side chain free to interact with LPS.

The present chapter demonstrates the application of polymyxin-cloth as a commercially attractive alternative to antibody-cloth for the quantitative and qualitative detection of LPS antigens. As a model system, the detection of LPS antigens from Salmonella cells was studied.

MATERIALS AND METHODS

Chemicals and immunoreagents

The following were obtained from Sigma Chemical Co.:

Salmonella typhimurium LPS (No. L-6511); E. coli strain 0128:B12 LPS (No. L-2755); sodium deoxycholate (No. D-6750); sodium caprylate (No. C-2875); and polymyxin B sulfate (No. P-1004). TMB microwell peroxidase substrate system (No. 50-76-00) and TMB membrane peroxidase substrate system (No. 50-77-00) were obtained from Kirkegaard and Perry Laboratories (KPL), Inc.. Buffered peptone was from Difco Laboratories.

Antibody used to coat the polyester cloth was a commercial preparation of polyclonal antibody (CSA-1) to heat-killed Salmonella cells (KPL, Inc., No. 01-91-99). The conjugate was CSA-1 antibody-horseradish peroxidase (KPL, Inc., No. 04-91-99), which was stored as a 0.1 mg/ml stock in PBS at -20 °C. For use, it was diluted 1:2000 in PBST.

Preparation of Salmonella antigens

Salmonella typhimurium strain LT2 stored at 4 °C on a nutrient agar slant was inoculated into buffered peptone water (BPW) and grown to a density of about 10^9 cells/ml (late log phase) by shaking at 37 °C for 16 h. The culture was diluted with BPW to obtain suspensions with various cell densities, which were confirmed from viable counts obtained by plating serial dilutions of the suspensions on nutrient agar and incubating the plates at 37 °C for 20 h. The

antigen for the CEIA was prepared by mixing 1 ml of fresh cell suspension with 0.1 ml of EDTA (0.5 M)-PBS (chapter 5), and heating the mixture at 100 °C for 10 min. Also tested in the CEIA were various preparations of S. typhimurium LPS dissolved in EDTA (0.05 M)-PBS and heated as above. The cooled mixtures were used immediately in the CEIA.

Cloth-based enzyme immunoassay (CEIA)

Nonwoven polyester cloth (DuPont, Sontara 8100) was cut into 6 mm square segments and coated with polymyxin or antibody. All incubations were performed in a closed petri dish at room temperature. To determine the optimal polymyxin concentration for coating, polyester cloth segments were incubated for 16 h with 50 µl of various concentrations of polymyxin B sulfate in PBS, and then washed 5 times with a total of about 5 ml of PBST on a macroporous filter under suction. The coated segments were then incubated with 50 µl of either 0 (negative controls) or 5×10^6 EDTA-heat-treated Salmonella cells per cloth for 30 min. The captured antigen was detected with the antibody-peroxidase conjugate as described below. For antibody coating, each cloth segment was incubated with 50 µl of the CSA-1 antibody (50 µg/ml in PBS) for 16 h, and then washed

as above. The coated cloths were stored in PBS at 4 °C until use. Polymyxin-cloth was stable for at least 3 months when stored under these conditions, and at least one month when stored in distilled water at room temperature.

For the CEIA, each polymyxin- or antibody-coated cloth segment was incubated with 50 µl of EDTA-heat-treated Salmonella sample for 30 min, then placed on an absorbent pad (disposable diaper), and washed 5 times dropwise with a total of about 0.5 ml of PBST. The segments were then incubated with 50 µl of the CSA-1 antibody-horseradish peroxidase conjugate for 30 min and washed as above. Peroxidase was assayed by placing each cloth in a 16 X 100 mm test tube and shaking in 1.0 ml of 3,3',5,5'-tetramethylbenzidine (TMB) microwell peroxidase substrate system for 60 min. The developed substrate solution was then transferred from the test tube to a 1 ml-capacity cuvette (1 cm light path) and its absorbance at 370 nm (A_{370}) was determined.

Dot blot assay

Polyester cloth was cut into 4 cm square sheets and each sheet was coated with 2 ml of polymyxin B sulfate (5 mg/ml in PBS), and then washed with 30 ml of PBST as above.

Fifteen μ l samples of EDTA-heat-treated Salmonella cell suspensions were pipetted on the polymyxin-cloth sheets at 1 cm intervals. After 30 min, each sheet was washed with PBST on a macroporous filter under suction, incubated with 2 ml of the CSA-1 antibody-peroxidase conjugate (in a petri dish) for 30 min, and then washed with PBST as above. Peroxidase was detected by soaking each sheet in 2 ml of TMB membrane peroxidase substrate system for 60 min. The sheets were washed with H₂O and examined for blue spots (which appeared against a white background) characteristic of the bound peroxidase activity. These sheets could then be stored at room temperature as a permanent record.

Determination of LPS removal from solution by polymyxin-cloth

Polyester cloths (6 mm square segments) were coated with 50 μ l of polymyxin B sulfate (5 mg/ml in PBS) as before, then washed with 5 ml of PBS on a macroporous filter. Ten segments of polymyxin-cloth were then incubated with 1 ml solutions of S. typhimurium LPS dissolved in PBS at various concentrations for 60 min. LPS in the solutions was assayed by determining the total sugar content before and after incubation with the polymyxin-cloth by the phenol-

sulfuric test for neutral glycoses of Dubois et al. (1956).

RESULTS

Effect of polymyxin concentration on the CEIA signal

The effect of using different polymyxin concentrations for coating polyester cloth on the capture of Salmonella antigens in an EDTA-heat-treated cell suspension was examined by the CEIA. The EDTA-heat treatment was used here in order to dissociate the LPS antigens from the cell surface (chapter 5), which should maximize the interaction of the LPS molecules with the polymyxin adsorbed on the cloth. Fig. 6.1 shows that the maximum CEIA signal (A_{370} value) was obtained when 6 mm cloth squares were coated with 4-6 mg/ml of polymyxin. Thus, subsequent experiments were performed using polymyxin at a coating concentration of 5 mg/ml. The LPS antigens released by the EDTA-heat treatment bound significantly to the uncoated cloth squares (no polymyxin), probably due to the hydrophobic lipid A moiety of the LPS. However, LPS binding to the polymyxin-cloth squares is largely due to the interaction between the LPS antigens and the adsorbed polymyxin. The negative controls (no antigen) exhibited consistently low background signals at all polymyxin concentrations tested.

Fig. 6.1. Effect of polymyxin concentration on Salmonella antigen detection by CEIA. Polyester cloth squares were incubated with various concentrations of polymyxin for 16 h, and the coated squares were then reacted with either 0 (O) or 5×10^6 (●) EDTA-heat-treated Salmonella cells. Antigen captured on the squares was detected using the antibody-enzyme conjugate as described in Methods. The CEIA signals (A_{370} value) are plotted as mean value $\pm$ standard error (n=4).

Kinetics of polymyxin adsorption onto polyester

In conventional EIA procedures, maximal adsorption of capture antibodies onto non-porous solid phases (e.g., microtiter plates) requires at least 16 h. Since polymyxin B is much smaller its adsorption to large-surface macroporous adsorbents such as polyester cloth should be faster. The kinetics of polymyxin adsorption at 5 mg/ml was examined by the CEIA of Salmonella antigens as above. Fig. 6.2 shows that polymyxin adsorption was indeed rapid, requiring only 2 h to produce coated cloth suitable for the CEIA. The CEIA signal showed only slight increases for periods longer than 2 h. In subsequent experiments, 6 h polymyxin coating was used. At higher polymyxin concentrations the coated cloth could be prepared in even shorter times (data not shown).

Kinetics of antigen capture

We have previously shown that the binding of Salmonella LPS antigens to specific antibodies adsorbed onto polyester cloth is rapid, requiring only 5-10 min for near-completion (chapter 5). The kinetics of Salmonella LPS antigen binding to the polymyxin-cloth was examined by detecting the antigen with the antibody-peroxidase conjugate as above. Fig. 6.3

Fig. 6.2. Kinetics of polymyxin adsorption onto polyester cloth. Polyester cloth squares were incubated with a 5 mg/ml solution of polymyxin for various periods of time, and the coated squares were then tested in the CEIA using 5×10^6 EDTA-heat-treated Salmonella cells per square as described in Methods. The CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error (n=4).

Fig. 6.3. Kinetics of antigen capture by polymyxin-cloth. Polymyxin-cloth squares were incubated with 5×10^6 EDTA-heat-treated Salmonella cells for various periods of time, and the captured antigen was then detected using the antibody-enzyme conjugate as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error ($n=4$).

shows that 25-30 min were required for near-completion of antigen capture, though shorter times such as 5-10 min were sufficient to obtain measurable signals in the CEIA. This slower antigen capture suggests that polymyxin-cloth has a lower affinity for the antigen than the antibody-cloth previously used.

Sensitivity of the polymyxin-CEIA

The sensitivity of the CEIA using polymyxin-cloth (polymyxin-CEIA) was compared to the CEIA using antibody-cloth (antibody-CEIA). Polyester cloth squares were coated with either polymyxin or anti-Salmonella antibody (CSA-1). The coated squares were incubated with various concentrations of EDTA-heat-treated Salmonella cells and the captured antigens were detected with the antibody-peroxidase conjugate as above. Fig. 6.4 shows that the antibody-CEIA provided about 10-fold higher sensitivity than the polymyxin-CEIA. The limit of detection was about 10^6 cells/ml for the antibody-CEIA and about 10^7 cells/ml for the polymyxin-CEIA. This difference is likely due to the polymyxin having a lower affinity for the LPS antigen than the CSA-1 antibody has. The CSA-1 antibody preparation contains not only LPS-specific antibodies, but also

Fig. 6.4. CEIA response to various antigen concentrations using polymyxin- and antibody-cloths. Polyester cloth squares coated with either polymyxin (●) or antibody (○) were incubated for 30 min with EDTA-heat-treated Salmonella suspensions containing various cell concentrations. The captured antigen was then detected using the enzyme-antibody conjugate as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error (n=4).

antibodies which recognize other heat-stable Salmonella antigens. This may partly account for the higher sensitivity of the antibody-CEIA for Salmonella cells.

Specificity of the polymyxin-CEIA

Since the cyclic peptide portion of polymyxin binds to the LPS of almost all Gram-negative bacteria (Vaara and Vaara, 1983), the binding site for the antibiotic must reside within a highly conserved region of the LPS molecule, such as the lipid A moiety. To examine whether the lipid A moiety of the LPS is involved in the interaction with polymyxin-cloth, S. typhimurium LPS was subjected to mild acid hydrolysis (described in chapter 4), which is known to remove the lipid A region (Hancock and Poxton, 1988). The removal of lipid A completely eliminated the ability of the antigen to produce a signal in the polymyxin-CEIA, even at higher concentrations of the antigen (>10 µg/ml), whereas in the antibody-CEIA the same hydrolyzed preparation produced about 80 % of the signal obtained using intact LPS. This suggests that an attached lipid A region is necessary for the capture of the LPS antigen by polymyxin-cloth, but not for its capture by antibody-cloth. At least a part of the driving force for the binding of lipid A to the polymyxin on

the cloth surface presumably involves hydrophobic interaction.

Since the lipid A region appears to be necessary for the binding of LPS to polymyxin, other substances having hydrophobic moieties may interfere with the LPS-polymyxin interaction when present in antigen samples. Therefore, we examined the effect of deoxycholate and caprylate (two model hydrophobic compounds) on the polymyxin-CEIA of Salmonella LPS. Table 6.1 shows that these compounds caused little change in the CEIA signal (in fact, the presence of deoxycholate in the sample seems to have slightly enhanced the signal). This indicates that the binding of lipid A to the polymyxin-cloth is probably not simply a non-specific hydrophobic interaction.

Since the LPS of other Gram-negative bacteria which might be present as contaminants of antigen samples will compete for binding to polymyxin, the detection of Salmonella in a sample containing these bacteria requires that the LPS binding capacity of the polymyxin-cloth be very high or that Salmonella LPS be present in significant proportions in the sample. We measured the amount of Salmonella LPS removed from solution by polymyxin-cloth at various LPS concentrations during a 60 min incubation

Table 6.1. Effect of deoxycholate and caprylate on the polymyxin-CEIA of Salmonella LPS ^a

Agent	<u>S. typhimurium</u> LPS (µg/ml)	A ₃₇₀ ^b
none	0	0.05 ± 0.0
	10	0.80 ± 0.06
sodium deoxycholate	0	0.06 ± 0.0
	10	0.85 ± 0.05
caprylic acid	0	0.05 ± 0.0
	10	0.78 ± 0.06

^a EDTA-PBS buffer containing 0 or 10 µg/ml of S. typhimurium LPS and 0 (no agent) or 100 µg/ml of either sodium deoxycholate or sodium caprylate was heated at 100 °C for 10 min, then cooled and subjected to the polymyxin-CEIA as described in Methods.

^b mean A₃₇₀ value ± standard error (n=3).

period, within which adsorption will be complete (Fig. 6.3). Table 6.2 shows that the amount of LPS removed from solution increased with increasing LPS concentrations. During the 60 min incubation a maximum of about 220 µg of LPS could be removed from a 1 ml solution by 10 polymyxin-cloth segments, which translates into about 22 µg of LPS per segment. This indicates that polymyxin-cloth has a high capacity for binding LPS.

Since the polymyxin-cloth exhibited a high LPS binding capacity, it should be possible to detect Salmonella LPS in the presence of LPS from other bacteria. Fig. 6.5 shows that the polymyxin-CEIA permitted the detection of Salmonella LPS in the presence of various concentrations of E. coli LPS. Even when the E. coli LPS was present in a 100-fold excess the polymyxin-cloth squares captured sufficient Salmonella LPS to give a significant CEIA signal. Samples lacking Salmonella LPS produced negligible signals at all E. coli LPS concentrations tested, thus confirming the specificity of the polymyxin-CEIA (which is imparted by the CSA-1 antibody-enzyme conjugate).

Table 6.2. Removal of LPS from solution by polymyxin-cloth ^a

LPS concentration ($\mu\text{g/ml}$) ^b		Total LPS removed from solution
Before	After	(μg)
50	40	10
100	60	40
200	80	120
400	210	190
800	580	220

^a Ten polymyxin-cloth squares (6 mm squares) were incubated for 60 min with 1 ml of PBS containing S. typhimurium LPS dissolved at various concentrations. The amount of LPS removed from the solutions was then determined as described in Methods.

^b LPS concentration before and after incubation with 10 polymyxin-cloth squares.

Fig. 6.5. Effect of E. coli LPS on the detection of S. typhimurium LPS by the polymyxin-CEIA. EDTA-PBS buffer containing 0 (O) or 1 (●) µg/ml of S. typhimurium LPS and various concentrations of E. coli LPS was heated at 100 °C for 10 min, then cooled and subjected to the polymyxin-CEIA as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error (n=4).

Applications of the polymyxin-CEIA

The results presented in the preceding sections suggest that the polymyxin-CEIA can provide a quantitative assay for Salmonella in a culture containing as few as 10^7 cells/ml (Fig. 6.4). In the screening of foods for Salmonella organisms there is a need for the rapid identification of Salmonella antigens either in liquid cultures or as colonies on plates. The dot blot assay method involving large sheets of hydrophobic membranes such as nitrocellulose (Tijssen, 1985) or filter paper (Fletcher, 1987) onto which multiple samples of antigens are blotted provides a convenient format for the screening of food samples for pathogens by EIA. However, this method requires that after antigen adsorption onto the membrane or filter paper by hydrophobic interaction the remaining sites must be completely blocked with proteins to prevent non-specific adsorption of antibody-enzyme conjugate, thus adding to the time required to complete the assay. Since the antigen is only passively adsorbed (i.e., without the use of a capturing agent) the sensitivity of such a dot blot assay is usually poor, and relatively large quantities of antigen must be applied. Also, extraneous sample components such as proteins, fatty substances, etc., might compete for the binding sites on the solid phase

surface and therefore interfere with the adsorption of the antigen. Coating the large sheets with a capture antibody would improve sensitivity and eliminate interference, but the cost of doing so would be prohibitive. Furthermore, the membrane is brittle, requiring care in handling a large sheet during transfer and washing. On the other hand, the use of polymyxin-cloth eliminates the need for a blocking step since the binding sites have already been saturated with polymyxin, and the presence of a capturing agent on the cloth should permit the sensitive detection of the LPS antigens with little or no interference from extraneous sample components. Furthermore, large sheets of polyester cloth are structurally stable and easy to wash because of the cloth's macroporosity. We therefore examined the use of polymyxin-cloth in a dot blot assay for the qualitative detection of Salmonella antigens.

Samples (15 μ l) of EDTA-heat-treated S. typhimurium cell suspensions of various cell densities were spotted at 1-cm intervals on a 4 cm square polymyxin-cloth sheet. After 30 min, the sheet was washed, incubated with a saturating volume of the antibody-peroxidase conjugate for 30 min, and developed in the peroxidase TMB substrate for 60 min. Suspensions containing 5×10^7 cells/ml produced weak

but definite colour reactions (blue spots against a white background), whereas suspensions containing 5×10^8 cells/ml or more produced highly visible colour reactions. An entire colony (2 mm diameter) of S. typhimurium grown on a plate was suspended in 1 ml of EDTA-PBS, heated at 100 °C for 10 min, and then cooled. Samples (15 µl) of such a suspension produced intense blue spots on the polymyxin-cloth sheet. Controls containing no Salmonella or samples with fewer than 5×10^7 cells/ml gave no colour.

DISCUSSION

These experiments demonstrate the suitability of polymyxin-cloth as a specific adsorbent for Salmonella LPS in its assay. Although the polymyxin-CEIA using EDTA-heat-treated samples was less sensitive than the antibody-CEIA, it should permit the quantitative and qualitative assay of LPS antigens at levels likely to be encountered in the identification of Salmonella in liquid cultures or on plates. Since the polyester cloth offered a large surface for the adsorption of polymyxin, the presence of non-Salmonella Gram-negative bacteria in test samples (such as enrichment cultures from foods) does not interfere significantly with the capture of Salmonella LPS. As

compared to the use of antibodies in EIA, the polymyxin-CEIA is not only simple, rapid and economical, but also should give highly reproducible results. Polymyxin was at least 100 times less expensive to use in the CEIA than commercial antibody preparations such as CSA-1. Furthermore, polymyxin is stable and does not suffer from batch-to-batch variations as do antibody preparations. The use of polymyxin as a capturing agent is particularly well suited for coating large sheets of cloth for qualitative dot blot assays. These features make the polymyxin-CEIA attractive for field tests involving large numbers of samples. Polymyxin-cloth also efficiently adsorbed the LPS antigens of other Gram-negative bacteria, such as several E. coli serotypes and Brucella abortus (data not shown). Therefore, the polymyxin-CEIA should be equally applicable to the assay of these bacteria, and other Gram-negative food pathogens such as Campylobacter, provided that suitable antibody-enzyme conjugates are available.

In the polymyxin-CEIA described here, antigen samples were prepared by heating in the presence of EDTA to extract the LPS in order to maximize its interaction with the polymyxin adsorbed onto the cloth surface. However, the kinetics of LPS capture and the sensitivity of this assay

were inferior to that of the antibody-CEIA, suggesting that LPS prepared in this manner has a lower affinity for polymyxin-cloth than for antibody-cloth. Alternative methods for preparing the antigen, such as the use of detergents for LPS extraction, may improve the sensitivity of the polymyxin-CEIA. The following chapter examines this possibility using the detection of Salmonella antigens as a model system.

CHAPTER 7

USE OF DETERGENTS IN THE PREPARATION OF SALMONELLA SAMPLES
FOR ENZYME IMMUNOASSAY ON POLYMYXIN-COATED POLYESTER CLOTH

ABSTRACT

Heating Salmonella cells in the presence of detergents such as deoxycholate or Triton X-100 greatly increased the sensitivity of an enzyme immunoassay for lipopolysaccharide antigens using polymyxin-coated polyester cloth as the solid phase for antigen capture. The presence of a 100-fold excess of E. coli lipopolysaccharide in the test sample did not affect the detection of the Salmonella lipopolysaccharide. The sensitivity of the assay using deoxycholate-heat treatment of the antigens followed by detection on polymyxin-cloth was equivalent to that of an assay using antibody-coated polyester cloth for antigen capture.

INTRODUCTION

In chapter 5 it was shown that heating Salmonella cells in the presence of ethylenediaminetetraacetate (EDTA) dissociates its antigens into non-sedimentable forms which can be readily assayed by EIA on antibody-coated polyester cloth (antibody-cloth). The use of polyester cloth as an immunoabsorbent for EIA provides a greater surface area for immunoreactions (thus, faster reactions) than non-porous solid phases such as microtiter plates, and also allows for

the concentration of dissociated antigens by filtration, which permits the detection of dilute antigens.

Chapter 6 demonstrated the use of polymyxin B-coated polyester cloth (polymyxin-cloth) for the capture of LPS antigens for the CEIA. As an LPS capturing agent, polymyxin is more advantageous than antibody in that it is much less expensive, more stable and is available in a highly homogeneous form. However, the EDTA-heat extracted LPS antigens assayed on polymyxin-cloth gave a lower CEIA signal than when assayed on antibody-cloth, suggesting that the affinity of these LPS antigens for the polymyxin is lower than for the antibody. It was also demonstrated that the lipid A moiety of the LPS molecule plays an essential role in its interaction with polymyxin-cloth. It is possible that the EDTA-heat treatment did not sufficiently dissociate the LPS antigen complexes to allow for the efficient interaction of the lipid A region (or a portion thereof) with the polymyxin-cloth. Therefore, an alternative method for the preparation of antigen samples was sought.

The ability of certain detergents to solubilize biological membranes is widely exploited in the preparation of membrane components such as lipids and proteins from prokaryotic as well as eukaryotic cells. Mild detergents

such as sodium deoxycholate, members of the Triton series and sodium dodecyl sulfate (SDS) are ideal for this purpose since these amphiphiles readily complex with the hydrophobic regions of membrane components and extract them from the hydrophobic environment of the lipid bilayer into the aqueous phase, usually without adversely affecting their salient properties (i.e., enzyme activity, antigenicity, etc.) (Helenius and Simons, 1975). The chemical structures of some of the more commonly used detergents are shown in Fig. 7.1. Sodium deoxycholate and SDS have been particularly useful for the mild dissociation of LPS complexes in studies of the outer membrane of Salmonella typhimurium and various other Gram-negative bacteria (Hannecart-Pokorni et al., 1973). Therefore, treating Salmonella cells with either of these detergents may extract the LPS in a form which can interact more efficiently with the polymyxin-cloth, resulting in the improved polymyxin-CEIA of the LPS antigens. This possibility will be examined in the present chapter.

(A)

(B)

(C)

(D)

[Sum of w, x, y, z is 20]

Fig. 7.1. Detergents commonly used in membrane studies.

(A) sodium deoxycholate, (B) Triton X-100, (C) sodium dodecyl sulfate, and (D) Tween 80.

MATERIALS AND METHODS

Chemicals and immunoreagents

The following were obtained from Sigma Chemical Co.: Salmonella typhimurium lipopolysaccharide (LPS) (No. L-6511), Escherichia coli serotype O128:B12 LPS (No. L-2755), polymyxin B sulfate (No. P-1004), sodium deoxycholate (No. D-6750), Triton X-100 (No. T-6878), sodium dodecyl sulfate (SDS) (No. L-5750), and Tween 80 (No. P-1754). TMB microwell peroxidase substrate system (No. 50-76-00) and a polyclonal anti-Salmonella (CSA-1) antibody (No. 01-91-99) and CSA-1 antibody-peroxidase conjugate (No. 04-91-99) were from Kirkegaard and Perry Laboratories, Inc.. The conjugate was stored as a 0.1 mg/ml stock in PBS at -20 °C. For use, it was diluted 1:2000 in PBST.

Preparation of Salmonella antigens

Salmonella typhimurium strain LT2 stored at 4 °C on a nutrient agar slant was inoculated into buffered peptone water (BPW) (Difco) and grown as described in chapter 6. The antigen for the EIA was prepared by mixing 1 ml of fresh cell suspension with 0.1 ml of either 1.1% (w/v) detergent in PBS or 0.55 M EDTA (pH 7.2) in PBS (EDTA-PBS). The mixture was then heated at 100 °C for 10 min, then cooled to

room temperature, and used immediately in the EIA.

Cloth-based enzyme immunoassay (CEIA)

Non-woven polyester cloth (DuPont, Sontara 8100) was cut into 6 mm squares which were wetted with PBS and then blotted. All incubations were performed in a closed petri dish at room temperature. The cloth was coated with polymyxin by incubating each square with 50 μ l of a 5 mg/ml solution of polymyxin B sulfate in PBS for 6-16 h, then washed with about 5 ml of PBST on a filter under suction. For antibody coating, each cloth square was incubated with 50 μ l of the CSA-1 antibody (50 ug/ml) for 16 h, then washed as above. The coated cloths were stored in PBS at 4 °C until use.

For the CEIA, each polymyxin- or antibody-cloth square was incubated with 50 μ l of either the EDTA-heat-treated or the detergent-heat-treated Salmonella samples for 30 min, then placed on an absorbent pad (disposable diaper) and washed 8 times dropwise with a total of about 1 ml of PBST. The squares were then incubated with 50 μ l of the CSA-1 antibody-peroxidase conjugate for 30 min, then washed with PBST as above. Unless stated otherwise, peroxidase was assayed by placing each square in a 16 X 100 mm test tube

and shaking in 1 ml of 3,3',5,5'-tetramethylbenzidine (TMB) microwell peroxidase substrate system for 30 min. Reactions were stopped by the addition of 0.1 ml of 0.5 M NaF, and the developed substrate was then transferred to a 1 ml-capacity cuvette (1 cm light path) and its absorbance at 370 nm (A_{370}) was determined.

EIA on a microtiter plate

Each well of a 96-well microtiter plate (Bio-Rad Labs, No. 224-0096) was coated with polymyxin by incubation with 200 μ l of a 5 mg/ml solution of polymyxin B sulfate in PBS for 16 h, then washed 8 times with PBST. All incubations were carried out at room temperature. For the EIA, each well was then incubated with 50 μ l of detergent-heat-treated LPS sample for 30 min. The wells were then washed with PBST as above, and incubated with 50 μ l of the CSA-1 antibody-peroxidase conjugate for 30 min, then washed again with PBST. Peroxidase was assayed by incubating each well with 0.3 ml of TMB microwell peroxidase substrate system for 30 min. The developed substrate solution was then transferred to a 16 X 100 mm test tube and diluted with 0.7 ml of 0.5 M NaF, and its A_{370} was determined as before.

RESULTS

Use of detergents in the preparation of *Salmonella* antigens

The increased sensitivity of a CEIA using antibody-cloth for antigen capture (antibody-CEIA) resulting from the extraction of *Salmonella* LPS antigens by EDTA-heat treatment was demonstrated in chapter 5. This method of antigen preparation was also employed for the CEIA of *Salmonella* LPS antigens on polymyxin-cloth (polymyxin-CEIA), although it gave a lower sensitivity than the antibody-CEIA (chapter 6). In order to improve the sensitivity of the polymyxin-CEIA, an alternative method of solubilizing *Salmonella* antigens using detergents was examined.

A suspension of *S. typhimurium* in BPW was mixed with one of a variety of detergents dissolved in PBS at different concentrations. The detergents tested were sodium deoxycholate, SDS, Tween 80, and various members of the Triton X series (X-15, X-45, X-100, X-165, and X-705). The detergent sample mixtures were then heated at 100 °C for 10 min, then used as sources of antigen for the polymyxin-CEIA. Fig. 7.2 shows that deoxycholate at 0.1% (w/v) (final sample concentration) produced the highest CEIA signal (A_{370} value) among the detergents tested. Triton X-100 produced a somewhat lower signal at the same optimum concentration, and

Fig. 7.2. Comparison of different detergents in the preparation of Salmonella antigens for the polymyxin-CEIA. A suspension of S. typhimurium cells (5×10^6 cells/ml) was mixed with 0.1 volume of either deoxycholate (O), Triton X-100 (●), SDS (Δ), or Tween 80 (▲) to give different final concentrations in the sample. The samples were then heated at 100 °C for 10 min, and subjected to the polymyxin-CEIA as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error (n=4).

other members of the Triton X series failed to produce enhancement at any concentration tested (data not shown), as did Tween 80. SDS produced a slight enhancement at a concentration of 0.01% (w/v) in the sample.

To compare the effect of deoxycholate treatment with that of the previous EDTA treatment, S. typhimurium cells suspended in BPW were treated with various combinations of deoxycholate, EDTA and heat. The mixtures were then tested as sources of antigen for the polymyxin-CEIA and the antibody-CEIA. Table 7.1 shows that heating the Salmonella cells in the presence of deoxycholate with or without EDTA produced the highest CEIA signal on both the polymyxin- and the antibody-cloths. The combined deoxycholate and heat treatment produced an approximate 10-fold increase in the polymyxin-CEIA signal compared to untreated samples. Heating in the presence of EDTA alone gave the maximum signal on antibody-cloth, but less than 30% of this signal on polymyxin-cloth. Heating the samples was required for maximum enhancement of the CEIA signals for the EDTA and deoxycholate treatments. However, deoxycholate treatment in the absence of heat produced an approximate 5-fold increase in the signal obtained on polymyxin-cloth compared to no treatment. Negative controls (no antigen) showed negligible

Table 7.1. Effect of deoxycholate, EDTA and heat treatments on the polymyxin-CEIA and the antibody-CEIA of Salmonella antigens ^a

Treatment			A_{370} ^b	
Heat	EDTA	Deoxycholate	Polymyxin-cloth	Antibody-cloth
+	+	+	0.52 ± 0.03	0.48 ± 0.05
+	-	+	0.51 ± 0.03	0.50 ± 0.04
+	-	-	0.12 ± 0.01	0.35 ± 0.02
+	+	-	0.15 ± 0.01	0.56 ± 0.04
-	+	-	0.07 ± 0.02	0.25 ± 0.03
-	+	+	0.25 ± 0.03	0.23 ± 0.03
-	-	+	0.23 ± 0.03	0.15 ± 0.02
-	-	-	0.05 ± 0.0	0.13 ± 0.01

^a Samples of Salmonella (5×10^6 cells/ml) in BPW were treated with various combinations of deoxycholate (0.1%), EDTA (50 mM, and heat (100 °C, 10 min), and then assayed by either the polymyxin- or the antibody-CEIA as described in Methods. +, treatment; -, no treatment.

^b Mean A_{370} value ± standard error (n=4).

3

of/de

3

signals ($A_{370} < 0.05$) with all treatments on the polymyxin- and antibody-cloths (data not shown).

In order to determine whether heating the cells in either deoxycholate or Triton X-100 extracted the LPS antigens into non-sedimentable forms, detergent- or EDTA-heat-treated S. typhimurium cells were centrifuged at 10 000 X g for 10 min, and the resulting supernatants were assayed by the polymyxin-CEIA as before. Approximately 80-90% of the total antigenic activity (as determined by the polymyxin-CEIA) was found in the supernatant of each treated sample. This indicates that all of these treatments caused the dissociation of the cell-associated antigens into non-sedimentable forms about equally. Therefore, the large enhancement of the polymyxin-CEIA by deoxycholate-heat-treatment of the antigen samples may involve more than simply a kinetic advantage conferred by the dissociation of the antigens into smaller units.

Detection of Salmonella LPS in the presence of E. coli LPS

Since polymyxin-cloth is generally specific for the LPS of Gram-negative bacteria, the presence of non-Salmonella LPS in a test sample will interfere with the capture of Salmonella LPS. However, in chapter 6 it was shown that

polyester cloth adsorbs enough polymyxin on its large surface to permit the capture of sufficient EDTA-heat-treated Salmonella LPS in the presence of E. coli LPS for detection by the polymyxin-CEIA. To test whether the same holds true for deoxycholate-heat-treated samples, solutions of S. typhimurium LPS were mixed with a solution of E. coli LPS so that the latter was present in either a 10- or a 100-fold excess. The LPS samples were then treated with deoxycholate-heat and assayed by the polymyxin-CEIA. Table 7.2 shows that the CEIA signal was reduced by approximately 23% when Salmonella LPS was assayed in the presence of a 100-fold excess of E. coli LPS, and by approximately 17% in the presence of a 10-fold excess of E. coli LPS, as compared to the CEIA signals obtained with Salmonella LPS samples lacking E. coli LPS. The E. coli LPS assayed in the absence of Salmonella antigens produced a slight CEIA signal above the background, and this is probably due to the presence of minor cross-reactive antibodies in the conjugate. Thus, the polymyxin-CEIA using deoxycholate-heat treatment was able to detect the Salmonella LPS with only a small reduction in the CEIA signal in the presence of excess E. coli LPS.

A polystyrene microtiter plate was also tested as the solid phase for the immobilization of polymyxin for use in

Table 7.2. Detection of Salmonella LPS in the presence of E. coli LPS. ^a

LPS in sample (µg/ml)		A ₃₇₀ ^b	
<u>S. typhimurium</u>	<u>E. coli</u>	Polymyxin-cloth	Polymyxin-plate
1	0	0.79 ± 0.04	0.54 ± 0.04
1	100	0.65 ± 0.04	0.15 ± 0.02
10	0	1.15 ± 0.06	0.55 ± 0.04
10	100	0.98 ± 0.05	0.41 ± 0.03
0	0	0.05 ± 0.0	0.03 ± 0.0
0	100	0.08 ± 0.0	0.03 ± 0.0

^a A solution of S. typhimurium LPS (0, 2 or 20 µg/ml) in BPW was mixed with an equal volume of BPW containing 0 or 200 µg/ml of E. coli LPS. The samples were then mixed with 0.1 volume of 1.1% deoxycholate and heated at 100 °C for 10 min, then assayed by EIA on polymyxin-cloth or a polymyxin-coated microtiter plate (polymyxin-plate). For the polymyxin-CEIA, peroxidase was assayed by placing each cloth square in an empty well of a microtiter plate and incubating with 0.3 ml of substrate for 30 min. The developed substrate was then removed from the wells and diluted into 0.7 ml of 0.5 M NaF, and its A₃₇₀ was determined as described in Methods.

^b Mean A₃₇₀ value ± standard error (n=4).

the EIA of LPS antigens. However, since the wells of the non-porous microtiter plate offer only a limited surface area for the adsorption of polymyxin, the presence of E. coli LPS in the sample may interfere to a greater extent with the capture of Salmonella LPS. Table 7.2 shows that the EIA signal for deoxycholate-heat-treated Salmonella LPS assayed on the polymyxin-coated microtiter plate (polymyxin-plate) suffered an approximately 72% decrease in the presence of a 100-fold excess of E. coli LPS, and an approximately 27% decrease in a 10-fold excess of the same. Thus, an excess of E. coli LPS in the sample produced more severe interference with the EIA of the deoxycholate-heat-treated Salmonella LPS on the polymyxin-plate than on polymyxin-cloth. This result confirms one important advantage of the high surface area of polyester cloth in EIA.

Kinetics of antigen capture

The kinetics of the capture of deoxycholate-heat-treated Salmonella LPS antigens on polymyxin-cloth and a polymyxin-plate were compared by means of the EIA. A suspension of S. typhimurium cells in BPW was treated with deoxycholate-heat, and then incubated with either polymyxin-

cloth or the wells of a polymyxin-plate for various periods of time. Bound antigen was then assayed using the peroxidase conjugate as above. Fig. 7.3 shows that the polymyxin-CEIA produced a significant enzyme signal ($A_{370} > 0.2$) within an approximately 2.5 min incubation with the antigen, whereas the polymyxin-plate EIA required at least 10 min to produce a significant signal. The figure also shows that neither the cloth nor the plate produced significant signals in the absence of polymyxin coating, thus confirming the essential role of polymyxin in antigen capture.

Sensitivity of the polymyxin-cloth EIA using deoxycholate

Here the sensitivity of the polymyxin-CEIA was determined using Salmonella samples heated in the presence of deoxycholate or EDTA. Suspensions of S. typhimurium in BPW at various cell densities were mixed with deoxycholate or EDTA-PBS, then heated and subjected to the polymyxin-CEIA as described in Methods. For comparison, samples heated in the presence of EDTA were also assayed by the antibody-CEIA. Fig. 7.4 shows that the limit of detection for the polymyxin-CEIA occurred in the range of 5×10^5 to 5×10^6 Salmonella cells/ml using deoxycholate-heat, and 5×10^6 to

Fig. 7.3. Kinetics of LPS capture. A suspension of S. typhimurium cells (5×10^6 cells/ml) in BPW was mixed with 0.1 volume of 1.1% deoxycholate and heated at 100 °C for 10 min. The sample was then incubated for various periods of time with either polymyxin-coated cloth (O), uncoated cloth (Δ), the wells of a polymyxin-coated microtiter plate ($\bullet$), or an uncoated microtiter plate ($\blacktriangle$). Bound antigen was then assayed by EIA as described in Table 7.3 legend. CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error (n=4).

Fig. 7.4. Sensitivity of the polymyxin-CEIA and the antibody-CEIA. Cell suspensions at various cell densities were mixed with 0.1 volume of either 1.1% deoxycholate (O) or 0.55 M EDTA-PBS (●,Δ). They were then heated at 100 °C for 10 min, and subjected to the CEIA on polymyxin-cloth (O,Δ) or antibody-cloth (●) as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ standard error (n=4).

5×10^7 cells/ml using EDTA-heat. The sensitivity of the polymyxin-CEIA using deoxycholate-heat-treated samples was similar to that of the antibody-CEIA using EDTA-heat.

DISCUSSION

These experiments demonstrate that heating Salmonella cells in the presence of certain detergents such as deoxycholate (and to a lesser extent Triton X-100) enhances the sensitivity of the CEIA using polymyxin-coated polyester cloth for the detection of LPS antigens, giving a level of sensitivity comparable to that achieved using antibody-coated polyester cloth and EDTA-heat treated cells (Fig. 7.4). The deoxycholate-heat treatment also enhanced the signal of the antibody-CEIA (as compared to an untreated sample), but not above the level achieved with the EDTA-heat treatment (Table 7.1). This, along with the observation that heating the samples in either deoxycholate, Triton X-100 or EDTA extracted the LPS antigens into non-sedimentable forms about equally, indicates that the enhancement of the polymyxin-CEIA using the detergent treatments involves more than simply a kinetic advantage conferred by the dissociation of the antigens into smaller units. While such a kinetic advantage does indeed exist, as is evident from

the enhanced antibody-CEIA signals obtained using the deoxycholate-heat treatment, complexing the LPS antigens with deoxycholate probably increases the affinity between the LPS molecules and the polymyxin-cloth. This is confirmed by the time course of antigen capture on the polymyxin-cloth, which shows that the reaction with LPS required about 5 min to reach completion using the deoxycholate-heat treatment (Fig. 7.3), whereas at least 15 min were required using EDTA-heat (Fig. 6.3).

The effectiveness of the different detergents in enhancing the interaction of the LPS antigens with the polymyxin-cloth was as follows (in the order of their decreasing effectiveness; Fig. 7.2): deoxycholate, Triton X-100, sodium dodecyl sulfate, and Tween 80. It is proposed that in order to enhance the interaction with the polymyxin-cloth the detergent must bind to the LPS molecule in such a way as to maximize the exposure of some site (which may be partly located within the lipid A region) which has an affinity for the polymyxin. Deoxycholate dissociates the LPS, but because of its bulky cyclopenthenophenanthrene ring structure (Fig. 7.1A) may not be capable of forming a tight complex, thus allowing the polymyxin access to binding sites located within the lipid A region of the LPS molecule.

Similarly, Triton X-100, because of its bulky aromatic ring (Fig. 7.1B), also forms 'loose' complexes with the LPS allowing for increased interaction with polymyxin. On the other hand, sodium dodecyl sulfate, with its compact aliphatic hydrocarbon tail (Fig. 7.1C), may form tighter complexes with the LPS thus obstructing sites in the lipid A region and diminishing its interaction with polymyxin. Tween 80 either complexes poorly with the LPS or its highly extended multi-branched structure (Fig. 7.1D) sterically hinders the interaction of LPS with the immobilized polymyxin.

Although the use of a microtiter plate as an adsorbent of polymyxin for the capture of deoxycholate-heat-treated Salmonella LPS antigens and their subsequent detection by EIA is feasible, the EIA signals obtained with the polymyxin-plate were generally lower than with polymyxin-cloth (Fig. 7.3). Furthermore, the capture of Salmonella LPS on the polymyxin-plate was subject to considerable interference from non-Salmonella LPS present in the sample, whereas polymyxin-cloth was not appreciably affected unless a large excess of non-target LPS was present (Table 7.2). This difference is due to the large surface area of the cloth, which permitted the immobilization of a greater

quantity of polymyxin.

The deoxycholate-heat treatment enables the more effective application of polymyxin-cloth to the qualitative detection of salmonellae in preparations such as enrichment cultures from food samples. For example, the improved sensitivity of the polymyxin-CEIA will enable the detection of antigens in enrichment cultures containing lower cell densities as a result of either the presence of competing microorganisms or the use of shorter incubation periods in faster tests. The polymyxin-CEIA in combination with the present method of deoxycholate-heat treatment for sample preparation should also be applicable to the detection of other Gram-negative bacteria, provided that suitable antibody-enzyme conjugates are available. Preliminary studies indicate its applicability to the detection of Campylobacter and Brucella LPS antigens in enrichment cultures. The following chapter will examine the application of polymyxin-cloth to the detection of salmonellae in foods using a semi-quantitative dot blot procedure involving large sheets of polymyxin-cloth able to accommodate multiple samples of deoxycholate-heat-treated antigens.

CHAPTER 8

DOT BLOT PROCEDURE FOR THE DETECTION AND SEMI-QUANTITATION OF SALMONELLA IN FOODS

ABSTRACT

A semi-quantitative dot blot assay for salmonellae in foods was developed. Various food samples inoculated with different numbers of Salmonella cells were homogenized in enrichment broth, then serially diluted in broth, and shaken at 37 °C for 20 h. The cultures were then heated at 100 °C for 10 min in 0.1% deoxycholate and spotted on a polymyxin-coated polyester cloth sheet for the capture of lipopolysaccharide antigens. The captured antigens were then detected by an anti-Salmonella antibody-peroxidase conjugate. This procedure distinguished different levels of Salmonella contamination in the food samples and required less than 24 h to complete.

INTRODUCTION

Meat processing industries are obliged to assay for Salmonella contamination in raw materials and finished products before use or sale. Rapid assays for Salmonella will reduce the deterioration of product quality, and the inventory costs incurred pending such assays. Rapid assays should also permit the early prevention and control of salmonellosis, which is very costly in terms of human misery, lost productivity, medical treatment, recall of

products, damage suits and confirmation of the disease. Currently, the rapid detection of viable Salmonella cells in food samples by culture methods involves the inoculation of sample material into a suitable liquid pre-enrichment medium (e.g., nutrient broth or buffered peptone water) to allow for the recovery of potentially damaged cells, followed by transfer of the sample to a selective enrichment medium (e.g., selenite cysteine or tetrathionate broths) to allow for the outgrowth of the salmonellae while suppressing non-Salmonella organisms, the entire procedure requiring a minimum of 48 h to complete (Andrews, 1985). The salmonellae in the pre-enrichment or selective enrichment cultures can then be identified either by (1) enzyme immunoassay (EIA) (Swaminathan et al., 1985), (2) biochemical and/or serological tests (Sperber and Deibel, 1969), or (3) a combination of these methods (Anderson and Hartman, 1985). EIA procedures can take the form of (1) a solid phase assay employing an anti-Salmonella antibody-coated solid phase (such as a polystyrene microtiter plate) for antigen capture, followed by detection of the captured antigen on the plate using an anti-Salmonella antibody-enzyme conjugate (Swaminathan et al., 1985), or (2) an immunological membrane filter method where a small volume of

pre-enriched culture is vacuum-filtered through a membrane filter (e.g., nitrocellulose or polysulfone filters) which is then placed on the surface of a selective agar plate to allow for the development of colonies, which are then identified by incubation with an anti-Salmonella antibody-enzyme conjugate (Cerqueira-Campos et al., 1986). All of these procedures give purely qualitative results, since they involve the pre-enrichment of the salmonellae in the original samples. Information regarding the extent of Salmonella contamination may be useful in controlling the spread of the organism, particularly in the poultry industry which suffers from a high prevalence of Salmonella contamination of its products (Stavric, 1987).

The present chapter describes a 24 h procedure for the semi-quantitative assay of viable Salmonella cells in food samples. In this procedure, a food sample is first homogenized in a liquid enrichment medium, then serially diluted in the enrichment medium, and then incubated to allow for the growth of the organisms. The cultures are then heated with deoxycholate (chapter 7) and tested for the presence of Salmonella antigens by a dot blot CEIA using large sheets of polymyxin-coated polyester cloth able to accommodate multiple samples (chapter 6). Using such a

procedure, it should be possible to distinguish between different levels of contamination in food samples on the basis of the minimum dilution which is negative for the salmonellae after the enrichment step.

MATERIALS AND METHODS

Chemicals and immunoreagents

Polymyxin B sulfate (No. P-1004) and sodium deoxycholate (No. D-6750) were obtained from Sigma Chemical Co.. CSA-1 anti-Salmonella antibody-peroxidase conjugate (No. 04-91-99), TMB microwell peroxidase substrate system (No. 50-76-00) and TMB membrane peroxidase substrate system (No. 50-77-00) were from Kirkegaard and Perry Laboratories, Inc.. The conjugate was stored as a 0.1 mg/ml stock in PBS at -20 °C. For use, it was diluted 1:4000 in PBST. Buffered peptone, yeast extract, selenite cysteine, tetrathionate broth base, and nutrient broth were from Difco.

Preparation of food samples

Food samples examined include fresh chicken breast meat, lean beef, aged cheddar cheese, unsweetened solid baker's chocolate, homogenized milk, mayonnaise and flour,

all of which were purchased from a local supermarket. Chicken and beef meats were blended to a paste using a food processor (5 X 10 second high speed bursts) prior to inoculation with Salmonella cells. For dry solid foods such as the cheese and solid baker's chocolate, one gram of the samples was blended in 2 ml of enrichment broth (10 X 10 second high speed bursts). Powdered, liquid and semi-solid food samples were used without blending.

Culture methods

Salmonella typhimurium strain LT2 stored on a nutrient agar slant at 4 °C was grown in buffered peptone water (BPW) and then diluted in BPW to obtain various cell densities, and used immediately for inoculation into food samples. BPW supplemented with 0.5% (w/v) yeast extract (BPWYE) was used as an enrichment medium for culturing Salmonella in the food samples.

One gram of food sample was inoculated with 1 ml of BPW containing various numbers of Salmonella cells. BPWYE was then added to the sample to a final volume of 10 ml, and the mixture was then homogenized thoroughly by vortexing. The homogenate was then serially diluted by transferring 0.5 ml into 4.5 ml of BPWYE in culture tubes (2.5 X 15 cm) to

obtain serial 10-fold dilutions. The tubes were then shaken at 37 °C for approximately 20 h, and then tested for the presence of Salmonella cells by the dot blot polymyxin-CEIA.

Dot blot polymyxin-EIA

Polyester cloth (DuPont, Sontara 8100) was cut into 4 X 6 cm sheets, then saturated with 3 ml of polymyxin B sulfate (5 mg/ml in PBS) and incubated for 16 h at room temperature. Each sheet was then washed with a total of about 6 X 10 ml of PBST on a filter under suction. For comparison, a dot blot EIA was also carried out on a 4 X 6 cm sheet of nitrocellulose (Schleicher and Schuell, No. 33172) coated with polymyxin as above.

One ml aliquots of enrichment culture were then mixed with 0.1 ml of 1.1 % (w/v) deoxycholate, and then heated at 100 °C for 10 min. Ten microliters of the deoxycholate-heat-treated samples were then pipetted onto the polymyxin-cloth or polymyxin-nitrocellulose sheets at 1-cm intervals, so that each 6 X 4 cm sheet accommodated a total of 24 regularly spaced samples. The sheets were then incubated for 30 min at room temperature, and then washed with PBST as above. Salmonella LPS antigens captured on the sheets were then detected by saturating each sheet with 1 ml of the CSA-

1 antibody-peroxidase conjugate and incubating for 30 min at room temperature. The sheets were washed with PBST, then blotted, and bound enzyme was detected by incubating each sheet with 1.5 ml of TMB membrane peroxidase substrate system for 10 min, which forms an insoluble coloured product upon reaction with the peroxidase. Areas on the cloth or membrane to which Salmonella LPS had bound produced distinct blue spots against a white background.

RESULTS

Comparison of Dot blot polymyxin-EIA on polyester or nitrocellulose

Currently, nitrocellulose membranes are the most commonly used solid phase for performing dot blot assays. Therefore, it was necessary to compare the performance of polyester cloth with a nitrocellulose membrane in the dot blot polymyxin-EIA. Salmonella cells suspended in BPWYE at various cell densities were treated with deoxycholate, and 10 µl samples were then blotted onto a cloth or membrane sheet with or without prior polymyxin coating. Salmonella antigens captured on the sheets were then detected using the CSA-1-peroxidase conjugate followed by developing in the peroxidase TMB substrate. Table 8.1 shows that coating

polyester cloth with polymyxin resulted in the detection of Salmonella in suspensions containing as few as 10^6 cells/ml, whereas the use of uncoated cloth only enabled the detection of between 10^7 and 10^8 cells/ml. This difference in sensitivity further demonstrates the advantage of using polymyxin adsorbed to the cloth for the capture of the deoxycholate-heat-treated LPS antigens. Antigen capture on polymyxin-cloth resulted in the formation of smaller, more intense and well defined coloured spots on the cloth, whereas uncoated cloth produced larger and more diffuse spots. This shows that the polymyxin binds tightly to the deoxycholate-heat-treated LPS antigens. The uncoated nitrocellulose membrane produced only faint colour reactions even at 10^8 cells/ml. Polymyxin-coated nitrocellulose detected 10^6 cells/ml but produced only a weak colour reaction at 10^7 cells/ml. Therefore, polymyxin-cloth was used in subsequent experiments for the dot blot assay of salmonellae in enrichment cultures from food samples. The sensitivity of the dot blot polymyxin-CEIA is necessary to permit the effective detection of Salmonella LPS antigens even in the presence of non-Salmonella Gram-negative bacteria originating from the food samples (chapters 6 & 7).

Table 8.1. Dot blot EIA on polyester cloth and a nitrocellulose membrane ^a

Solid phase	<u>Salmonella</u> cells/ml				
	10 ⁸	10 ⁷	10 ⁶	10 ⁵	0
Polyester cloth					
coated	+	+	+	-	-
uncoated	+	+/-	-	-	-
Nitrocellulose					
coated	+	+/-	-	-	-
uncoated	+/-	+/-	-	-	-

^a 4 X 6 cm sheets of polyester cloth or a nitrocellulose membrane were either coated with polymyxin or left uncoated, and 10 ul samples containing different concentrations of deoxycholate-heat-treated Salmonella cells in BPWYE were assayed by the dot blot EIA on the sheets as described in Methods. +, strong colour reaction (dark blue spot) ; +/-, weak colour reaction (faint blue spot); -, no colour reaction.

Assay of Salmonella in food samples

One of the main concerns of food processors is the determination of the absence or presence of salmonellae at any level in foods. Any reduction in the total time required to detect the organisms in samples by culture methods will be of great value to the food industry. Therefore, a simple 24 h test for detecting salmonellae in foods using a combination of enrichment culture and the dot blot polymyxin-CEIA was examined. BPWYE was employed as an enrichment medium, since it was observed that S. typhimurium exhibited a shorter lag period in this medium at 37 °C than when grown in BPW alone under similar conditions.

One gram quantities of food samples were inoculated with various numbers of S. typhimurium cells and shaken in BPWYE for approximately 20 h at 37 °C. The cultures were then treated with deoxycholate and assayed for Salmonella LPS antigens by the dot blot polymyxin-CEIA. Table 8.2 shows that these cultures (undiluted) permitted the detection of at least 10 Salmonella cells originally inoculated in each of the food samples, and at least 1 cell wherever tested. When assaying the enrichment cultures from chocolate samples, it was necessary to dilute the cultures 1:10 in PBS before performing the dot blot assay.

Otherwise, a negative dot blot result was sometimes obtained in the inoculated cultures. Presumably, some fatty component of the chocolate sample interfered with the assay. No such dilution was necessary for the other food samples tested. This combined enrichment culture-dot blot polymyxin-CEIA should be useful for the rapid (i.e., within 24 h) qualitative assay of salmonellae in food samples.

In some instances, it would be desirable to assess the extent of Salmonella contamination in certain foods (such as poultry products), necessitating quantitative or semi-quantitative rather than qualitative methods. There are currently no simple, rapid procedures for accomplishing this. If samples containing different levels of Salmonella cells are serially diluted, eventually a dilution will be reached which lacks cells, since the distribution of the cells over the dilution series must follow a Poisson distribution. The Poisson distribution states that the probability (P_0) of a given dilution failing to receive a single cell increases with decreases in the average number of cells (m) present in the sample, according to the equation, $P_0 = e^{-m}$. Therefore, it should be possible to distinguish between different levels of contamination in

food samples on the basis of the minimum dilution in a series which fails to receive even a single cell.

Food samples inoculated with different numbers of Salmonella cells and then suspended in BPWYE were serially diluted in BPWYE, then shaken for approximately 20 h at 37 °C. The cultures were then assayed for Salmonella LPS antigens by the dot blot polymyxin-CEIA. Table 8.2 shows that food samples containing higher levels of inoculated Salmonella cells required greater dilutions (i.e., endpoints) in order to produce Salmonella-negative cultures. However, since the endpoint determinations are dependent on the achievement of cell distributions according to the Poisson distribution, they may not be reliable as a quantitative means of assessing the extent of contamination in the original samples. Nonetheless, the method clearly permitted the distinction of 100-fold differences in the level of Salmonella contamination of the samples, and therefore appears to be suitable as a semi-quantitative procedure. Greater statistical reliability in the endpoint determination can be achieved by carrying out repetitive serial dilutions of the original sample suspensions, if desired.

Table 8.2. Dot blot polymyxin-CEIA of salmonellae in foods^a

Food sample	Cells/ml ^b	Dilution factor					
		undiluted	10	10 ²	10 ³	10 ⁴	10 ⁵
None	1000	+	+	+	+	+	-
(BPWYE)	100	+	+	+	-	-	-
	10	+	+	-	-	-	-
	1 ^c	+	-	-	-	-	-
	0	-	-	-	-	-	-
Chicken meat	1000	+	+	+	+	-	-
(boneless	100	+	+	+	+	-	-
breast)	10	+	-	-	-	-	-
	1 ^c	+	-	-	-	-	-
	0	-	-	-	-	-	-
Beef meat	1000	+	+	+	+	-	-
(lean)	100	+	+	+	-	-	-
	10	+	+	-	-	-	-
	1 ^c	+	-	-	-	-	-
	0	-	-	-	-	-	-

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Table 8.2 (continued)

Milk	1000	+	+	+	+	-	-
(2% fat)	100	+	+	+	-	-	-
	10	+	-	-	-	-	-
	1 ^c	+	-	-	-	-	-
	0	-	-	-	-	-	-
Mayonnaise	1000	+	+	+	+	-	-
(Kraft)	100	+	+	+	+	-	-
	10	+	+	-	-	-	-
	1 ^c	+	-	-	-	-	-
	0	-	-	-	-	-	-
Flour	1000	+	+	+	+	-	-
(wheat)	100	+	+	+	-	-	-
	10	+	-	-	-	-	-
	0	-	-	-	-	-	-

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Table 8.2 (continued)

Cheese	1000	+	+	+	+	+	-
(Kraft)	100	+	+	+	-	-	-
	10	+	+	-	-	-	-
	1 ^c	+	-	-	-	-	-
	0	-	-	-	-	-	-
Chocolate	1000	+	+	+	+	+	-
(Baker's)	100	+	+	+	+	-	-
	10	+	+	-	-	-	-
	0	-	-	-	-	-	-

^a One gram of blended food sample was inoculated with 1 ml of BPWYE containing various numbers of S. typhimurium cells, and sterile BPWYE was added to a final volume of 10 ml. The suspension was thoroughly mixed and then serially diluted in sterile BPWYE to give serial 10-fold dilutions of the original suspension, and the cultures were then incubated as described in Methods. Salmonella cells were then assayed in the original (undiluted) and dilution cultures by the polymyxin-CEIA dot blot as described in Methods. Dot blot results: +, colour reaction (blue spot); -, no colour reaction (no spot).

Table 8.2 legend continued on following page...

Table 8.2 legend (continued)

^b Final density of inoculated Salmonella cells in the original sample suspension after adjustment of the volume to 10 ml.

^c Samples inoculated with a calculated average of one cell may not consistently produce positive results, since the probability of the failure to receive even a single cell is approx. 0.37 (according to the Poisson distribution).

Effect of mechanical blending on the detection of viable Salmonella cells in foods

In order to ensure a homogeneous distribution of the Salmonella cells for dilution, it was necessary to disrupt solid or semi-solid food samples (e.g., meat, cheese, etc.) by means of mechanical blending. In the preceding experiments, the samples were homogenized by using a food processor with repeated high speed bursts of short duration, and were then inoculated with known numbers of Salmonella cells. However, in naturally contaminated foods the cells would be present before disruption of the samples, and it was therefore necessary to determine whether blending solid

samples containing live cells causes extensive injury which could markedly affect their assay by the present method.

A 10 g sample of solid cheese was suspended in BPWYE and blended to a liquid paste. The paste was then inoculated with various numbers of S. typhimurium cells and thoroughly mixed. A sample (unblended cells) was then withdrawn and the remaining inoculated cheese was further blended in the food processor, and a second sample (blended cells) was withdrawn. The two samples were then mixed with BPWYE and then serially diluted in sterile BPWYE and cultured for approximately 20 h. The cultures were then assayed by the dot blot polymyxin-CEIA. Table 8.3 shows that blending did not affect the ability of the assay to distinguish between the different levels of Salmonella cells inoculated in the cheese samples. Thus, mechanical blending appears to be suitable as a means of disrupting sample solids for the assay.

Table 8.3. Effect of mechanical blending on salmonellae assayed by the dot blot polymyxin-CEIA ^a

Cells/ml ^b	Dilution factor					
	undiluted	10	10 ²	10 ³	10 ⁴	10 ⁵
Unblended						
1000	+	+	+	+	-	-
10	+	+	-	-	-	-
0	-	-	-	-	-	-
Blended						
1000	+	+	+	+	+	-
10	+	-	-	-	-	-
0	-	-	-	-	-	-

^a A cheese sample (10 g) was blended in 15 ml of BPWYE using a food processor (10 X 10 second high speed bursts), and then inoculated with 1 ml of BPWYE containing different numbers of S. typhimurium cells and mixed. Samples (2 ml) from this mixture before (unblended) and after (blended) blending in a food processor (10 X 10 second high speed bursts) were adjusted to 10 ml with BPWYE and then serially diluted in BPWYE, then cultured. The cultures were then assayed by the dot blot polymyxin-CEIA as described in Methods. Dot blot results: +, colour reaction (blue spot); -, no colour reaction (no spot).

^b Final density of Salmonella cells in the sample suspension after adjustment of the volume to 10 ml.

Polymyxin-CEIA of Salmonella LPS antigens in other enrichment media

The enrichment medium (BPWYE) employed in these experiments gave very satisfactory results for the culture of the S. typhimurium cells in the various food samples tested. With the exception of chicken meat, these food samples apparently contained very few non-Salmonella organisms capable of growth in this medium: the uninoculated food samples failed to develop turbidity when the starting sample suspensions were diluted beyond 1:1000. However, uninoculated chicken meat developed turbidity after the overnight incubation at all of the dilutions of the original sample suspension tested, indicating the presence of large numbers of non-Salmonella contaminants. For foods heavily contaminated with non-Salmonella organisms, it may be necessary to use selective media such as selenite cysteine and tetrathionate broths (Patil and Parhad, 1986) for the culture of natural Salmonella isolates. These media are usually employed after pre-enrichment of the samples using non-selective broths such as unsupplemented buffered peptone water (Ibrahim and Lyons, 1987) and nutrient broth (Cerqueira-Campos et al., 1986). Depending on the requirements of the test, the enrichment procedure can be

simplified by using a non-selective broth alone, as in the present experiments. Since all of these enrichment broths are useful for the culture of salmonellae from foods, it was necessary to determine whether they interfere with the assay of Salmonella LPS antigens by the dot blot polymyxin-CEIA.

S. typhimurium LPS was dissolved in various enrichment media which were then treated with deoxycholate and assayed by the polymyxin-CEIA using polymyxin-coated polyester cloth segments. Table 8.4 shows that the assay on polymyxin-cloth was able to detect the LPS antigens equally effectively in all of these media (as judged by the CEIA signals). Thus, the polymyxin-CEIA should be applicable to the detection of salmonellae in these and possibly other enrichment media. When multiple samples must be assayed, as in the dilution culture procedure described in this chapter, the dot blot format should be suitable for the procurement of qualitative results.

Table 8.4. Polymyxin-CEIA of Salmonella antigens in various enrichment media ^a

Medium	LPS	A_{370} ^b
	($\mu\text{g/ml}$)	
SCB	0	0.07 ± 0.0
	0.1	0.33 ± 0.04
TBB	0	0.06 ± 0.0
	0.1	0.35 ± 0.03
BPW	0	0.06 ± 0.0
	0.1	0.34 ± 0.03
BPWYE	0	0.05 ± 0.0
	0.1	0.37 ± 0.04
NB	0	0.06 ± 0.0
	0.1	0.32 ± 0.02

^a Samples of various enrichment media containing 0 or 0.1 $\mu\text{g/ml}$ of S. typhimurium LPS were treated with deoxycholate-heat and then assayed by the polymyxin-CEIA using 6 X 6 mm segments of polymyxin-cloth as described in chapter 7. The enrichment media tested were selenite cysteine broth (SCB), tetrathionate broth base (TBB), buffered peptone water (BPW), BPW supplemented with 0.5% (w/v) yeast extract (BPWYE), and nutrient broth (NB).

^b Mean A_{370} value $\pm$ standard error (n=4).

DISCUSSION

The results presented in this chapter demonstrate that a simple enrichment culture method combined with the dot blot polymyxin-CEIA permitted the detection of viable Salmonella cells in a variety of foods within 24 h. The entire procedure was sensitive, allowing for the detection of as few as 1-10 S. typhimurium cells inoculated into one gram of food sample. When combined with a method for preparing dilution enrichment cultures from food suspensions, the dot blot polymyxin-CEIA was useful in the semi-quantitative assay of the Salmonella cells. The determination of the endpoints (which are subject to the Poisson distribution) using the dilution culture-dot blot polymyxin-CEIA enabled the distinction between high and low levels of inoculation with S. typhimurium cells in all of the food samples tested. Mechanical blending of a cheese sample containing viable Salmonella cells had no appreciable effect on the ability of the method to distinguish between the different levels inoculated, suggesting that this treatment produced little or no injury to the cells. Therefore, mechanical blending is suitable for the preparation of solid food samples for dilution. The recovery of cells which may be entrapped within the matrix

of a solid food sample might be further improved by treatment with mild detergents to dissolve fatty or waxy material. In instances where non-Salmonella organisms such as other Enterobacteriaceae are present in food samples in large numbers, it may be necessary to follow the pre-enrichment in non-selective broth by a selective enrichment step in order to eliminate the competing microorganisms and increase the recovery of the salmonellae. There exist a variety of useful non-selective and selective enrichment broths for culturing Salmonella organisms in foods, and the polymyxin-CEIA was able to detect deoxycholate-heat-treated Salmonella LPS antigens efficiently in all of the most commonly used enrichment broths.

The polymyxin-CEIA is an efficient and sensitive assay for the detection of LPS antigens in a variety of samples such as food suspensions and enrichment media. The assay is adaptable to several useful formats, such as quantitative tests using individual cloth segments and qualitative dot blots on large sheets capable of accommodating multiple samples. The present thesis has examined the advantages of this macroporous cloth as an adsorbent for use in EIA, and several applications of these advantages in the detection of specific antibodies and antigens have been demonstrated.

CHAPTER 9

AFFINITY PURIFICATION AND BIOTINYLATION OF ANTIBODIES

ON ANTIGEN-COATED POLYESTER CLOTH

ABSTRACT

The advantages of polyester cloth as an adsorbent of immunoreactants were exploited in the preparation of an immunoreagent for use in enzyme immunoassay. Anti-Salmonella antibodies in an antiserum were immunoadsorbed onto lipopolysaccharide-coated polyester cloth, biotinylated and then eluted. The result was a biotinylated affinity purified antibody which required less than 2 hours to prepare, and which could be used immediately in the detection of Salmonella antigens by enzyme immunoassay using a streptavidin-alkaline phosphatase conjugate.

INTRODUCTION

An extremely high affinity exists between avidin, a glycoprotein abundantly available from egg white, or streptavidin, a bacterial protein produced by Streptomyces avidinii, and the low-molecular-weight vitamin biotin. One molecule of avidin (or streptavidin) has four active binding sites for biotin (Bayer and Wilchek, 1974). Covalently coupling biotin to an antibody enables the biotinylated antibody to bind to avidin or streptavidin (Tijssen, 1985). Since many biotin molecules can be coupled to an antibody, a biotinylated antibody can bind multiple molecules of avidin.

When the avidin (or streptavidin) is coupled to an indicator such as an enzyme, these properties can provide an excellent means of linking the enzyme to specific antibodies for use as immunoreagents (Guesdon et al., 1979). Biotinylated antibodies and avidin- (or streptavidin-) enzyme conjugates have become a popular combination for the detection of antigens captured by immobilized antibodies in EIA since it provides greater sensitivity than direct antibody-enzyme conjugates (Hancock and Poxton, 1988), and a variety of avidin- and streptavidin- enzyme conjugates are commercially available. Biotinylated antibodies are usually prepared in the laboratory, and their preparation requires an intensive effort involving first the purification of the desired antibodies, followed by their biotinylation in free solution using a reactive biotin derivative, the unused portion of which must be removed from the reaction mixture after biotinylation is complete (Shamsuddin and Harris, 1983). Antibodies can be purified from antiserum by a variety of methods, including fractionation with salts, ion-exchange chromatography, affinity purification on a solid phase (e.g., Sepharose beads) to which antigens have been coupled, or a combination of these methods (Tijssen, 1985). The entire procedure, starting with the purification of the

antibodies and ending with their biotinylation, requires a few days to complete.

The present chapter describes a rapid and simple procedure which allows for both affinity purification and biotinylation of antibodies on antigen-coated polyester cloth. The advantages of polyester cloth as an immunoadsorbent for EIA have been described in previous chapters. Polyester cloth is inexpensive, provides a large surface area for immunoreagent adsorption, and can be easily washed after immunoreaction. These advantages should make antigen-coated polyester cloth suitable as an adsorbent for the affinity purification of specific antibodies from antiserum. Furthermore, biotinylation of immunoadsorbed antibodies directly on the cloth should be possible. Since the cloth can be easily washed after reaction, its use would eliminate the need for the dialysis or gel filtration steps which are required for the preparation of biotinylated antibodies in free solution. To demonstrate this, the biotinylation of anti-Salmonella antibodies from an antiserum on Salmonella lipopolysaccharide (LPS)-coated polyester cloth will be examined.

MATERIALS AND METHODS

Chemicals and immunoreagents

The following were obtained from Sigma Chemical Co.: biotinamidocaproate N-hydrosuccinimide ester (BACHS) (No. B-2643), Salmonella typhimurium LPS (No. L-6511), p-nitrophenyl phosphate (No. 104-0), bovine serum albumin (BSA) (No. A-7888), and anti-rabbit IgG antibody-alkaline phosphatase conjugate (No. A-8025). Streptavidin-alkaline phosphatase conjugate was from Boehringer Mannheim (No. 1089-161). Affinity purified polyclonal antibodies (CSA-1) to heat-killed Salmonella cells were from Kirkegaard and Perry Laboratories, Inc. (No. 01-91-99), as was CSA-1 antibody-alkaline phosphatase conjugate (No. 05-91-99). Before use, all conjugate stock solutions were diluted in PBST at the manufacturer's recommended working dilution, except for the streptavidin-alkaline phosphatase conjugate which was used at a dilution of 1:4000. The preparation of rabbit anti-Salmonella serum (antiserum) has previously been described in chapter 2. Normal rabbit serum was a pre-immunization serum devoid of anti-Salmonella antibodies.

Preparation of LPS-coated polyester cloth

Polyester cloth (DuPont, Sontara 8100) was cut into 6

mm square segments. Before coating, S. typhimurium LPS was dissolved in PBS containing 0.05 M EDTA (pH 7.2) (EDTA-PBS) and was then heated at 100 °C for 10 min. For use in affinity purification, 10 polyester cloth segments were incubated with 1 ml of the LPS solution (100 µg/ml) for 16 h at room temperature, then washed with PBST on a macroporous filter under suction. For use in EIA, each polyester cloth segment was incubated as above with 50 µl of EDTA-heat-treated LPS solution (10 µg/ml) in a closed petri dish, and then washed with PBST. The LPS-cloths were stored at 4 °C in PBS.

Affinity purification and biotinylation of antibodies

Ten LPS-cloth segments were blotted and incubated with 1 ml of anti-Salmonella serum in PBS for 30 min at room temperature. The segments were then washed with PBS and blotted. For biotinylation, the cloth segments were suspended in 1 ml of 0.01 M borate-buffered (pH 8.0)-0.85% NaCl (BBS) and mixed with 10 µl of a BACHS solution (10 mg/ml in dimethylformamide). After 30-60 min incubation at room temperature with occasional gentle stirring, the cloth segments were washed with PBS as above and blotted, and the bound biotinylated antibodies were eluted by shaking the

segments in 1 ml of 0.1 M glycine-HCl (pH 2.2) for 5 min at room temperature. The buffer containing the eluted sample was then removed to a clean test tube with a pipette and neutralized by the addition of 0.2 volume of 1.0 M Tris-HCl (pH 8.0). This solution could then be used immediately in the EIA of Salmonella antigens or stored at 4 °C for at least 2 weeks. For prolonged storage at -20 °C, we recommend dialyzing the solution against PBS and adding bovine serum albumin to a final concentration of 5% (w/v) as a stabilizer.

Protein assay

Proteins were measured using a commercial Coomassie protein assay (Pierce, No. 23201) which was not affected by Tris in the protein samples. The protein standard used was BSA.

Determination of antibody titers

Fifty microliters of serially diluted antibody in PBS were applied on LPS-cloth segments and then incubated for 30 min at room temperature in a petri dish. Each segment was then washed with PBST and then incubated with 50 µl of anti-rabbit IgG-alkaline phosphatase conjugate for 30 min at

above. After washing with PBST as above, each segment was incubated for 30 min at 37 °C with 0.5 ml of 15 mM p-nitrophenyl phosphate in 1.0 M diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl₂. The reaction was stopped by addition of 0.5 ml of 0.1 M Na₂HPO₄, and the absorbance at 404 nm (A₄₀₄) was determined. Titer values were determined as the maximum dilution factor which produced CEIA signal nearest an A₄₀₄ value of 0.2.

Test of biotinylated antibody in the CEIA of Salmonella antigens

S. typhimurium strain LT2 was grown by shaking in buffered peptone water (BPW) (Difco) at 37 °C to a density of about 10⁹ cells/ml, and was then diluted with BPW to various cell densities (determined by viable counts). To solubilize the antigens (chapter 5), the samples were mixed with 0.1 volume of 0.5 M EDTA in PBS (pH 7.2), heated at 100 °C for 10 min, then cooled to room temperature and used immediately for antigen assay by the CEIA.

Polyester cloth segments (6 mm squares) were incubated with 50 µl of CSA-1 antibody (50 µg/ml in PBS) for 16 h at room temperature and then washed with PBST. The antibody-coated cloth segments were then incubated with 50 µl of the

Salmonella samples (prepared as above) for 30 min at room temperature, and washed with PBST. The cloth segments were then incubated with 50 μ l of either biotinylated antibodies (0.05 μ g/ml in PBST) or the CSA-1 antibody-alkaline phosphatase conjugate (0.05 μ g/ml in PBST) for 30 min at room temperature, and then washed with PBST. The segments treated with biotinylated antibodies were further incubated with 50 μ l of the streptavidin-alkaline phosphatase conjugate for 30 min at room temperature, then washed with PBST. Each segment was then assayed for bound alkaline phosphatase activity by incubating with substrate as above.

RESULTS

Affinity purification of antibody on LPS-cloth

Salmonella LPS-coated polyester cloth was examined for its suitability as an immunoabsorbent for the purification of anti-Salmonella antibodies. Ten LPS-cloth segments were incubated with 1 ml of different dilutions of the antiserum and the amount of adsorbed protein eluted with a pH 2.2 buffer was measured. Fig. 9.1 shows that the amount of protein recovered was maximal when the antiserum was diluted up to 4 times, but beyond this the protein recovery decreased with increasing dilutions. This indicates that

Fig. 9.1. Recovery of total proteins from LPS-cloth.

Ten S. typhimurium LPS-coated polyester cloth segments (6 mm squares) were incubated with 1 ml of different dilutions of either antiserum (●) or normal serum (○) in PBS for 30 min. The cloths were then washed and the antibodies eluted with 1 ml of pH 2.2 buffer, then neutralized. The samples were then assayed for total proteins as described in Methods.

antibody binding sites on the LPS-cloth were saturated at an input serum dilution of 1:4 and lower. Incubating the LPS-cloth segments with normal serum resulted in only minimal recovery of proteins at the lower serum dilutions and no measurable recovery at serum dilutions of 1:8 and higher. This suggests that the proteins eluted from the LPS-cloth were mostly anti-Salmonella antibodies and that an antibody preparation free of non-specific serum proteins can be obtained when the antiserum is properly diluted prior to adsorption.

The recovery of the anti-Salmonella antibody titer during the affinity purification was examined by comparing the antibody titer eluted from the LPS-cloth with the original titer in the input antiserum solution. The antibody titer was determined in the CEIA by measuring the binding of the serially diluted antibodies applied to LPS-cloth segments with an anti-rabbit IgG-alkaline phosphatase conjugate. Since the pH 2.2 buffer used in elution caused a five-fold reduction in the capacity of the antibodies to complex with the conjugate (data not shown), it was necessary to first treat the anti-Salmonella antiserum with pH 2.2 buffer, and compare the titer of the treated antiserum with the titers of the pH 2.2-eluted samples. To

avoid prolonged exposure to pH 2.2 the elution was performed only once, although repeated elution would have increased the recovery. Fig. 9.2 shows that the recovery of anti-Salmonella antibody from the original antiserum was maximal (approximately 30%) using an input serum dilution of 1:8. Thus, subsequent experiments in which antibodies were biotinylated on the LPS-cloth were performed using an input serum dilution of 1:8.

To examine the reusability of the LPS-cloth, the same LPS-cloth was used four times for the affinity purification of anti-Salmonella antibodies. There was no appreciable loss in the recovery of purified antibodies during the four cycles of use.

Biotinylation of anti-Salmonella antibodies on LPS-cloth

Biotinylation of the antibodies while immunoabsorbed on the LPS-cloth should prevent biotinylation of the antigen binding sites on the antibody molecules, which may reduce the affinity of the antibody for the antigen. It should also simplify the procedure since unused reaction mixture can be readily removed by washing. Therefore, the conditions for biotinylating antibodies immunoabsorbed on LPS-cloth were studied by varying the pH, biotinylating

Fig. 9.2. Recovery of anti-Salmonella antibody titer in affinity purification. LPS-cloth segments were incubated with different dilutions of antiserum, and antibodies were eluted with pH 2.2 buffer then neutralized as in Fig. 9.1. The anti-Salmonella titers of the eluted samples were then measured by the CEIA as described in Methods. The recovery of the original titer of each antiserum dilution was determined as described in the text.

reagent concentration, and biotinylation time. The biotinylated antibodies were then eluted with pH 2.2 buffer and neutralized. The eluted biotinylated antibodies were reacted with Salmonella antigens captured on CSA-1 antibody-coated cloth segments to which 5×10^6 EDTA-heat-treated Salmonella cells had been applied. The bound biotinylated antibodies were then detected with a streptavidin-alkaline phosphatase conjugate as described in Methods. Fig. 9.3 shows that biotinylation (as judged by the CEIA signal) was maximal at a pH of 8.0-8.5 (Fig. 9.3A), and a biotinylating reagent (BACHS) concentration of 0.1 mg/ml (Fig. 9.3B) after a reaction time of 30-60 min (Fig. 9.3C). Therefore, subsequent biotinylation reactions were carried out using 0.1 mg/ml BACHS in BBS (pH 8.0) and a reaction time of 30 min. Under these conditions, the entire procedure (including the affinity purification) required less than 2 h to complete.

Performance of biotinylated antibodies in the CEIA

The biotinylated anti-Salmonella antibodies prepared directly on LPS-cloth (B-Ab I) were compared with affinity-purified anti-Salmonella antibodies biotinylated in free solution (B-Ab II) and a direct CSA-1 antibody-alkaline

Fig. 9.3. Conditions for the biotinylation of immunoadsorbed antibodies. Antibodies adsorbed to 10 LPS-cloth segments were biotinylated using 1 ml of (A) BACHS (0.2 mg/ml) suspended in either 0.01 M acetate (pH 6.5)-0.85% NaCl buffer, PBS (pH 7.0-7.5), or BBS (pH 8.0-9.5), and a reaction time of 60 min, (B) various concentrations of BACHS in BBS (pH 8.0) and a reaction time of 60 min, or (C) BACHS (0.1 mg/ml) in BBS (pH 8.0) and various reaction times. After pH 2.2 elution and neutralization, the biotinylated antibodies were tested in the CEIA of Salmonella cells as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ S.E. (n=4).

phosphatase conjugate (CSA-1-AP) in the CEIA of S. typhimurium antigens. B-Ab II was prepared from antiserum by affinity-purification on LPS-cloth as described in Methods, followed by extensive dialysis of the eluted antibodies against BBS and reaction with BACHS (0.1 mg/ml) for 30 min at room temperature, and then further dialysis against PBS to remove unreacted BACHS. This procedure required a total of 2 days to complete.

The biotinylated antibodies (B-Ab I and B-Ab II) used in combination with a streptavidin-alkaline phosphatase conjugate, and the CSA-1-AP conjugate, were tested in the CEIA of EDTA-heat-treated Salmonella cells using CSA-1 antibody-coated cloth as described in Methods. Fig. 9.4 shows that the limit of detection was about 10^6 Salmonella cells/ml using the B-Ab I system and about 5×10^6 cells/ml using the B-Ab II system or the CSA-1-AP conjugate. Thus, biotinylation of the immunoadsorbed antibodies resulted in a preparation which was slightly more sensitive in the CEIA of Salmonella antigens than the use of antibodies biotinylated in free solution or a commercial antibody-enzyme conjugate. The higher CEIA signals obtained with the B-Ab I system may be due to protection of the antigen binding sites of the antibodies against biotinylation.

Fig. 9.4. CEIA of Salmonella antigens. EDTA-heat-treated S. typhimurium suspensions containing various cell concentrations were incubated with CSA-1 antibody-coated cloth. The captured antigens were then detected using biotinylated antibodies (either B-Ab I (●) or B-Ab II (○)) in combination with a streptavidin-alkaline phosphatase conjugate, or a CSA-1 antibody-alkaline phosphatase conjugate (▲) as described in Methods. CEIA signals (A_{370}) are plotted as mean value $\pm$ S.E. (n=4).

DISCUSSION

These experiments demonstrate the application of LPS-coated polyester cloth in the affinity purification and biotinylation of anti-Salmonella antibodies. This method was not only rapid, simple and economical, but also resulted in the preparation of a biotinylated antibody which permitted the sensitive detection of Salmonella antigens by CEIA. The method should be applicable to the preparation of biotinylated antibodies not only to other Gram-negative bacteria (e.g., Campylobacter), but also to other hydrophobic antigens adsorbable to polyester cloth. In instances where antigens (e.g., some proteins) might be sensitive to the low pH exposure, alternative elution conditons may be required if repeated use of the antigen-cloth is desired. Since polyester cloth has excellent flow characteristics due to its macroporosity and non-compressibility, its use in a large scale column operation for the preparation of larger quantities of biotinylated antibodies should be feasible.

CONCLUSIONS

This thesis has examined the advantages of using macroporous hydrophobic cloths such as polyester cloth as immunoadsorbents for EIA, and several applications of these advantages in the detection of antibodies and antigens have been demonstrated. As compared to non-porous solid-phases such as microtiter plates, polyester cloth provides a large surface area for the immobilization of immunoreagents (antigens or antibodies) and permits rapid immunoreactions with the test sample (chapter 1). These advantages were exploited in several ways. The ability of polyester cloth to provide rapid immunoreactions was applied in the quantitative assay of specific antibodies on the basis of their initial rate of immunoreaction with antigen-coated cloth (chapter 2), thus eliminating the need for the lengthy incubation periods required in EIA performed on non-porous solid phases in order to obtain detectable immunoreactions. This feature also enabled the rapid assay of anti-Salmonella antibodies in chicken egg yolk (chapter 3), thus providing a promising method for monitoring sanitary conditions in poultry rearing facilities. The use of antigen-coated cloth should enjoy many other applications in the assay of antibodies. For example, antigen-cloth sheets capable of accommodating multiple samples should be useful in the rapid

screening of large numbers of hybridoma cultures for specific monoclonal antibodies using a dot blot format. They should also be useful in the screening of blood banks for the presence of specific polyclonal antibodies such as anti-hepatitis virus.

The large surface area of the cloth, combined with a simple method for extracting Salmonella cell surface antigens into non-sedimentable forms, also allowed for the rapid capture of the antigens by antibody-coated cloth in their EIA. This advantage made possible the use of antibody-coated cloth for the concentration of dilute Salmonella antigens present in large volumes of sample by rapid filtration, thereby increasing the effective sensitivity of the cloth-based EIA (CEIA) (chapter 5). The ability of antibody-coated cloth to concentrate dilute antigens present in large sample volumes should also be suitable for the sensitive detection of other analytes (e.g., trace pollutants in environmental samples such as water). When the analyte is a hapten bearing a single epitope, a 'blocking' EIA format (similar in principle to that illustrated in Fig. 2B) can be adopted, in which the analyte binds to specific antibodies on the cloth during concentration and therefore quantitatively blocks sites for

the subsequent interaction with an enzyme-labelled hapten. Such an approach might be extremely useful for the assay of environmental pollutants such as pesticides, dioxins or any substance against which specific antibodies can be prepared. It may also be useful in the assay of certain substances in biological samples, such as metabolites of drugs in urine or blood.

The polyester cloth also permitted the immobilization of polymyxin B for use as a capturing agent in the assay of LPS antigens (chapter 6). Because of its large surface area, sufficient polymyxin could be immobilized on the polyester cloth to allow for the detection of the target LPS even in the presence of excess non-target LPS in the sample. Heating Salmonella cell samples in the presence of certain detergents such as deoxycholate and Triton X-100 extracted the LPS antigens in a form which interacted more efficiently with the polymyxin-cloth, thus allowing for its use in the sensitive assay of the antigens (chapter 7). Because the cloth and polymyxin are inexpensive, large sheets of polymyxin-coated polyester cloth able to accommodate multiple test samples were used in a qualitative dot blot assay of Salmonella antigens in samples such as enrichment cultures. This convenient format for processing larger

numbers of samples was especially useful in the development of a semi-quantitative dilution culture-dot blot polymyxin-CEIA, which permitted the distinction between high and low levels of Salmonella cells in various inoculated food samples (chapter 8). Although the Salmonella problem addressed in this thesis is in itself an important area in which to endeavor, the polymyxin-CEIA should prove equally valuable for the detection of other Gram-negative bacteria, provided that suitable antibody-enzyme conjugates can be prepared. Since a large proportion of all bacterial pathogens are Gram-negative organisms, the polymyxin-CEIA has the potential of becoming an important tool for the diagnosis of infectious diseases. My preliminary experiments indicate its applicability to the detection of Campylobacter (now recognized as an important food pathogen) and Brucella organisms.

Finally, the high surface area of the cloth permitted its use as an immunoabsorbent for the affinity purification of antibodies in an antiserum. The antibodies could be coupled to biotin while immunoabsorbed to the cloth. This resulted in an affinity purified biotinylated antibody preparation which was useful as an EIA immunoreagent, and which was ready for use in only a small fraction of the time

normally required to prepare biotinylated antibodies by conventional methods.

Enzyme immunoassay is becoming increasingly popular as a tool for the diagnosis of infectious diseases and more generally as a method for the detection of a broad variety of analytes. Reports on its application to the detection of new substances now regularly appear in the literature. The development of macroporous hydrophobic solid phases such as polyester cloth opens many new practical possibilities for EIA as a diagnostic and analytical tool.

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