THE APPLICATION OF IMMUNOLOGY TO FOOD SCIENCE: TWO STUDIES:

PRODUCTION OF MONOCLONAL ANTIBODIES (Mabs) SPECIFIC FOR AN ENTEROPATHOGENIC E.COLI (EPEC); DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR β-N-ACETYLGLUCOSAMINIDASE (NAGase)

by

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ABSTRACT

Two hybridoma clones, labelled 4D10 C1 and 2H4 H12, produced monoclonal antibodies which recognized the outer membrane of an enteropathogenic Escherichia coli (EPEC) O142:K86:H6 in an enzyme-linked immunosorbent assay (ELISA) and the whole cell in an immunofluorescence assay. Large scale production of the monoclonal antibodies was accomplished through ascites production in balb/c mice. Purification of the ascites fluid was achieved by gel filtration and ion exchange chromatography. Isotyping of the purified fractions showed 4D10 C1 to be an IgG2 and 2H4 H12 an IgM. These monoclonal antibodies were screened by immunofluorescence assay against several pathogenic and non-pathogenic strains of E.coli in addition to other Enterobacteriaciae. Results of the screening showed these antibodies to be specific for the E.coli serotype to which they were raised. Minimal cross-reactivity with other Enterobacteriaceae was observed.

In a separate and concurrent project, the use of an ELISA capable of detecting ß-N-acetylglucosaminidase (NAGase) was examined. White Leghorn hens were injected with commercially prepared bovine NAGase. Eggs were collected and the immunoglobulin fraction separated from the egg yolk by polyethylene glycol precipitation followed by ion exchange on a DEAE-Sephacel column. The use of the purified immunoglobulins was examined in a sandwich, double-sandwich and a competitive ELISA. A statistically significant

standard curve for the detection of NAGase was successfully derived using a double-sandwich ELISA when rabbit immunoglobulin was used to coat the microwell plates. This assay was used to measure the NAGase concentration in press juice and fish extract of fresh and frozen salmon muscle samples. The ratio of the NAGase concentration in the press juice to the total NAGase concentration was compared. No significant difference was found between the calculated concentration ratios of the fresh muscle samples and samples frozen for 1 week at $-20^{o}C$.

TABLE OF CONTENTS

MATERIALS AND METHODS

PART I: MONOCLONAL ANTIBODY (Mab) STUDY

PART II: DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

LIST OF TABLES

LIST OF FIGURES

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INTRODUCTION

The immune system is a complex defense mechanism with an evolutionary history spanning 400 million years (Weir, 1988). This system includes all those physiological mechanisms that enable an animal to recognize materials that are foreign to itself, such as bacteria, and to neutralize or eliminate them without injury to itself (Bellanti and Kadlec, 1985).

Recognition of, and ultimately protection against, foreign substances is accomplished by immunoglobulins. Immunoglobulins are glycoproteins that are capable of binding to the infective agent, or antigen (Weir, 1988).

Immunoglobulins produced as a result of stimulation of the immune system are termed "polyclonal" due to their diversity in class, specificity for the antigen and biological function (Bankert et al., 1984). One of the most important advances in immunology has been the development of current monoclonal antibody production techniques. Monoclonal antibodies are homogeneous, having arisen from the clone of a single plasma cell (Bankert et al., 1984). These antibodies offer many advantages over conventional polyclonal antisera, including improved specificity for the antigen.

Monoclonal antibodies may be useful in the detection of enteropathogenic E.coli (EPEC). EPEC represent one of the major causative agents of diarrhea among infants in developing countries, resulting in a high incidence of morbidity (Black et al., 1981). While monoclonal antibodies have been raised against the virulence-associated antigens of

enterotoxigenic E.coli (Svennerholm et al., 1986), this remains to be done with EPEC strains. If developed, such a monoclonal antibody may have potential use in an immunoassay for EPEC in immunoglobulin infant feeding studies, food systems, and in diagnostic and epidemiological studies of causes of diarrhea in developing countries.

An immunoassay is based on the interaction between an antigen and its specific antibody (Pesce et al., 1978). The most popular immunoassay used today is the Enzyme-Linked Immunosorbent Assay (ELISA) first described by Engvall and Perlmann in 1971. The use of enzyme labels in immunoassays has allowed them to become more economical, safe and simple (Monroe, 1984). Due to their versatility, ELISAs are being used more widely among the scientific disciplines, including food science.

Attempts to develop a simple and reliable method to distinguish fresh from frozen/thawed fish fillets have not been successful. The use of an ELISA to detect the release of the lysosomal enzyme ß-N-acetylglucosaminidase during freezing of fish muscle may have potential use for this purpose.

The objectives of this research were to:

1. gain a knowledge of monoclonal antibody techniques.
2. produce a monoclonal antibody specific for enteropathogenic E.coli.
3. develop an ELISA for ß-N-acetylglucosaminidase which could be used to differentiate between fresh and frozen/thawed fish muscle.

LITERATURE REVIEW

A. Antibodies and the Immune System

1. The Immune System

The immune systems' responses can be narrowed down into two categories, the "Innate" or nonspecific responses and Specific Aquired Immunity. Nonspecific responses, while being capable of differentiating self from nonself, are not dependent on the specific recognition of a foreign configuration. Nonspecific immunity includes, for example, those first line barriers against infection such as the skin, phagocytosis of cells by macrophages and the inflammatory response (Roitt, 1988; Bellanti and Kadlec, 1985).

Specific Aquired Immunity is mediated by two types of mechanisms. They are Cell-mediated Immunity and Humoral Immunity. Cell-mediated Immunity is mediated by specifically sensitized lymphocytes that differentiate under the influence of the thymus and are called T-lymphocytes (Bellanti and Rocklin, 1985)

Humoral Immunity is mediated by a group of lymphocytes that are derived from the bone marrow (or bursa of Fabricus in birds) and are therefore called B-lymphocytes (Bellanti and Kadlec, 1985). The products of the humoral immune response are antibodies. Antibodies are complex proteins known as immunoglobulins which are capable not only of activating the complement system and stimulating phagocytic cells (Roitt, 1988), but also binding to and neutralizing substances foreign to the body. These substances are called

antigens.

2. Antibody Production

While the bone marrow is the source of B-lymphocytes, once produced they will travel to peripheral lymphoid tissues such as the lymph nodes, spleen and lymphoid tissue of the gastrointestinal and respiratory tracts (Bellanti and Kadlec, 1985). Each B-lymphocyte is genetically programmed to produce only one antibody species. This antibody is present on the outer surface of the lymphocyte and acts as a receptor (Roitt, 1988). Each lymphocyte has on the order of 10^5 antibody molecules on its surface. When a foreign substance (antigen) enters the body, it will bind to those antibody receptors which provide a "good fit". Once this binding takes place, the B-lymphocytes are stimulated to differentiate and proliferate into plasma cells. These plasma cells then secrete antibodies which are identical to the receptor antibody on the B-cell from which it was derived (Roitt, 1988), and therefore are capable of binding to the antigen.

3. Antibodies: Structure and Function

Antibodies belong to a class of proteins known as immunoglobulins. They are composed of four polypeptide chains bound by disulphide bonds (Guttman et al., 1981) as indicated in Figure 1. Intra-chain disulphide bonds influence the shape of the individual chains (Weir, 1988). The molecule consists of two identical light chains, each of

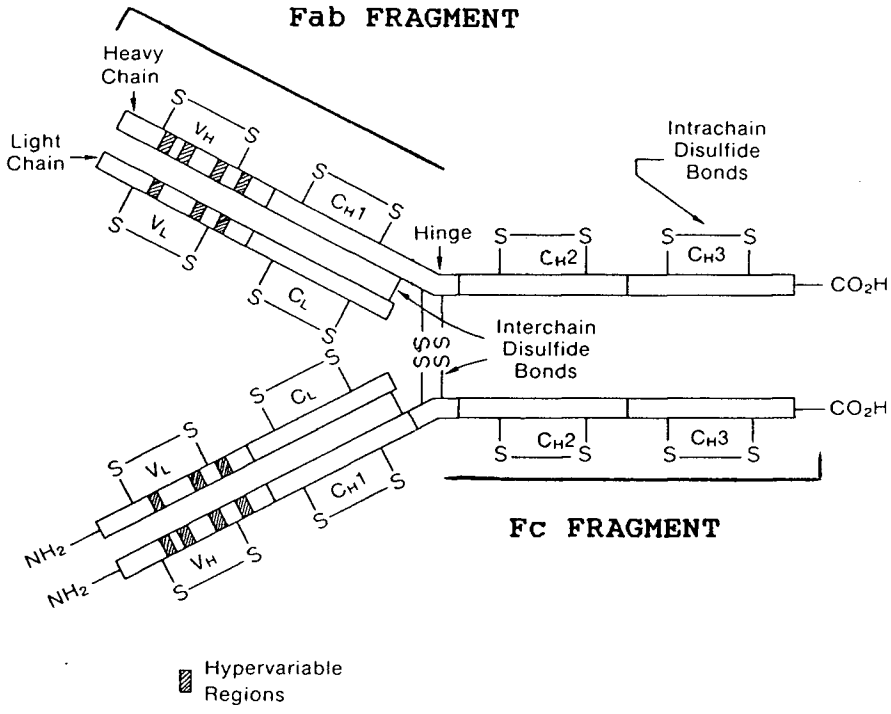

Figure 1: Basic structure of an antibody molecule (adapted from Calvanico, 1984)

which is 214 amino acids long, and two identical heavy chains which are between 450 and 700 amino acids long (Guttman et al., 1981).

Both the light chains and the heavy chains have variable and constant regions (Bellanti and Kadlec, 1985). In the light chains, the constant sequence of amino acids is located at the C terminal end of the chain. The variable region exists in the first 107 amino acids at the N terminal. Up to 50% of the positions in the N terminal have been found to be variable (Weir, 1988). This variability tends to be concentrated in three areas known as the hyperviariable regions (Weir, 1988) (Figure 1). Consequently, a large number of permutations are possible in sequence and therefore antibody specificity. Similar variation is seen in the N terminal of the heavy chain (Figure 1). It is in these variable portions of the heavy and light chains that the antigen-binding site is found (Weir, 1988). There are two antigen-binding sites per immunoglobulin molecule (Figure 1).

In humans, five different classes of immunoglobulins are known to exist (this differs from species to species) (Weir, 1988). These can be differentiated from one another on the basis of size, amino acid sequence, biological function and biochemical properties. These classes are known as IgG, IgA, IgM, IgD and IgE (Bellanti and Kadlec, 1985).

IgG is the major immunoglobulin in serum, accounting for 70-75% of the serum immunoglobulin. This immunoglobulin has the common structure already defined and has a molecular

weight of approximately 150,000 daltons (Bernier, 1985). IgG can also be broken down into subclasses based o differences in the Fc regions (Calvanico, 1984). There are four IgG subclasses: IgG1, IgG2, IgG3, and IgG (Spiegelberg, 1974).

IgA is the second most abundant immunoglobulin in the serum and is important in the external secretory system There are two IgA subclasses (IgA1 and IgA2) (Calvanico, 1984). IgA has a molecular weight of approximately 170,000 daltons (Bernier, 1985).

IgM is the largest of the immunoglobulin molecules. Five of the 4-chain structures common to immunoglobulin structure are joined together to form a large molecule of approximately 900,000 daltons (Bernier, 1985). There are two subclasses of IgM (IgM1 and IgM2) in man (Calvanico, 1984). This class of immunoglobulins is most important in the first few days of the primary immune response (Bernier, 1985).

IgD has a molecular weight of approximately 150,000 daltons (Bernier, 1985) and is commonly present in only trace amounts in serum. The biological function of IgD is not exactly known (Weir, 1988).

IgE has a molecular weight of approximately 196,000 daltons and is also present in only trace amounts in serum. IgE is responsible for triggering allergic reactions (Weir, 1988).

Antibodies can be broken down into three pieces by digestive enzymes. Two pieces are identical and carry the

antigen-binding site. They are called the Fab fragments (Figure 1). The third fragment is called the Fc portion. This fragment lacks the antibody binding site, but has many other important functions such as complement fixation (Weir, 1988).

4. Antigens and Antigen-Antibody Interaction

Substances which are capable of triggering an immune response are called immunogens. Antigens are substances which are capable of binding with a specific antibody (Pesce et al., 1978). An antigen alone may not be immunogenic. For example, some low molecular weight substances, called haptens, cannot stimulate the immune system unless first coupled to a larger carrier molecule (Weir, 1988).

Areas on the antigen such as a certain amino acid sequence or a sugar side chain are called antigenic determinants or epitopes. It is these sites that the antibody binds to. The part of the variable region on the antibody which binds with the epitope is called the paratope (Bernier, 1988).

An antibody is able to recognize an antigen based on its conformation (Weir, 1988). This is similar to the "lock and key" arrangement in enzyme-substrate interactions. The forces that bind the antigen and antibody together are common intermolecular forces such as electrostatic, hydrophobic and hydrogen bonding, as well as Van der Waal's forces. No covalent bonding is involved (Weir, 1988).

The specificity of an antibody for an antigen is not absolute. In other words, an antibody raised against a specific antigen may cross-react with a related antigen which either has an identical or similar antigenic determinant. When the antigen is similar, the "fit" between antigen and antibody will not be exact. This will result in weaker binding.

5. Chickens as a Source of Antibody

Conventional sources of antibodies or antisera include such animals as rabbits and mice. More recently, interest has been shown in the use of immunoglobulins from avian sources such as chickens and, in particular, chicken egg yolk.

Three major classes of antibody have been identified in birds. They are IgM, IgA and IgG (Rose and Orlans, 1981). Most researchers agree that yolk contains only IgG (Rose and Orlans, 1981). The IgG found in chicken egg yolk is commonly referred to as IgY due to differences from IgG in several characteristics, including molecular weight, sedimentation constant and isoelectric point (Leslie and Clem, 1969; Altschuh et al., 1984).

It has already been demonstrated that hens immunized with an immunogenic substance lay eggs containing large amounts of IgY specific for this substance (Polson et al., 1980; Berger et al., 1985) and that this immunoglobulin can be easily isolated from the egg yolk in relatively high yields (Burger et al.,1985). In addition, there is increased convenience in

the collection of eggs over the bleeding of animals. For this reason, the use of chickens represents a viable alternative for production of antisera.

B. Monoclonal Antibodies

1. History

Because of the heterogeneity or diversity among immunoglobulins in their structure, specificity and biological function, antiserum derived from an immunized animal is termed "polyclonal" (Bankert et al., 1984). The clonal selection theory (Burnet, 1957) postulates that each B-cell or plasma cell produces antibodies with a single specificity. Therefore, a clone from a single cell would secrete antibodies of uniform specificity. The term monoclonal is used to define a population of immunoglobulins that are identical; meaning that they have arisen from a single clone of a plasma cell. This property could not be utilized in vitro initially because B-cells and plasma cells cannot be maintained in tissue culture for any length of time.

In 1975, Kohler and Milstein devised a method whereby they could isolate a single plasma cell from an immunized mouse and make it "immortal" by fusing it with a myeloma cell. Since that time, the technology has come so far that it is reasonable to say that it is possible to raise monoclonal antibodies against any antigen that can stimulate an immune response.

2. Monoclonal Antibody Production

a. Basic Principle

When two cells are brought into close contact with one another and their membranes are caused to fuse together, the fusion product will contain both nuclei. Eventually the nuclei will fuse producing a hybrid; a cell which contains a single nucleus yet retains the genetic information from both of the original cells (Zola and Brooks, 1982). This principle forms the basis of the Kohler and Milstein (1975) monoclonal methodology which involves the fusion of murine myeloma cells and spleen cells derived from an immunized mouse. An example of present day monoclonal antibody production methodology is summarized in Figure 2.

b. Immunization

The first step in hybridoma production is the immunization of a suitable animal. The animals most commonly used for this purpose are rats and mice (Waldmann, 1986), since myeloma cell lines for these species are available commercially (Eshhar, 1985) and they are easily immunized. Interspecies hybridomas, such as rodent-human hybrids (Westerwoudt, 1986) or rodent-bovine hybrids (Guidry et al., 1986), can be produced. However, loss of chromosomes usually occurs as a result of fusion. This rarely occurs with rodent-rodent hybrids (Westerwoudt, 1986). While *in vivo* immunization is most common, immunization of the spleen *in vitro* is also possible (Sirvaganian et al., 1983). This approach can be used if the immunogen is toxic to the animal.

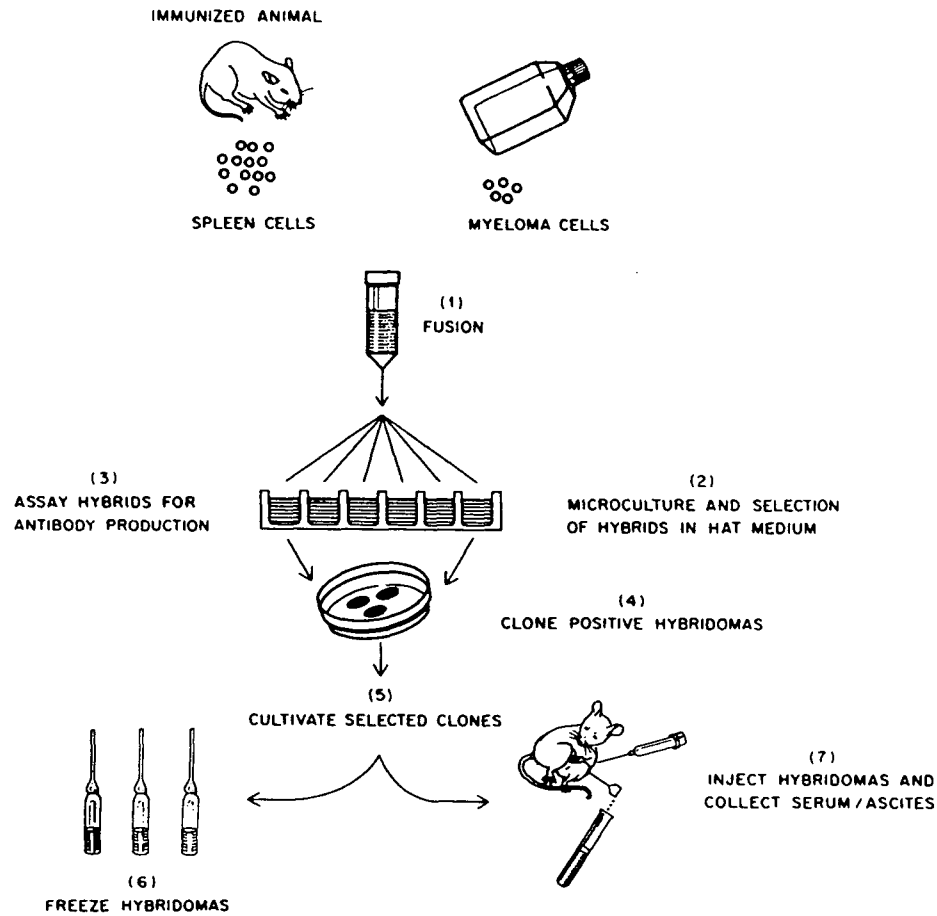

Figure 2: Outline of a common protocol used to produce hybridomas (Bankert et al., 1984)

c. Myeloma Cells and the HAT Selection System

In order to be suitable for monoclonal work, the myeloma cell line selected must possess a characteristic which makes it possible to selectively separate hybrids from the parent myeloma cells. A selective media is used which results in the death of myeloma cells, but allows hybrids to grow. The most commonly used media is HAT media.

The HAT media system was developed by Littlefield, 1964. HAT media contains hypoxanthine, aminopterin and thymidine. Aminopterin is a folic acid analog which blocks the main biosynthetic pathway of nucleic acid synthesis (Bankert et al., 1984). Cells can continue to grow by a salvage pathway if hypoxanthine and thymidine are present in the media. This salvage pathway can be utilized only if the cells contain the enzymes hypoxanthine guanine phosphoribosyltransferase (HGPRT) (Bankert et al., 1984) and thymidine kinase (Eshhar, 1985).

In the most common technique of monoclonal antibody production, myeloma cell mutants are selected which are deficient in either of these enzymes. Therefore, they cannot grow in the presence of aminopterin, even if hypoxanthine and thymidine are present in the media (Bankert et al., 1984). Hybrids formed between a spleen cell and a myeloma cell can grow because the spleen cell parent contains the necessary enzymes to utilize the salvage pathway. As was mentioned previously, spleen cells cannot be maintained in tissue culture; therefore, spleen cells and hybrids formed between

spleen cells die off naturally.

d. Fusion

The frequency of fusion of cell membranes is increased by the addition of fusing agents, often called fusogens (Zola and Brooks, 1982). The Sendai virus was the fusogen used in the first fusion experiments (Kohler and Milstein, 1975). This has given way to the use of more practical agents, the most popular being polyethylene glycol (PEG) (Galfre et al., 1977). The exact mechanism of action of the PEG is not known; however, it is largely thought to cause agglutination of the cells, thereby increasing the area of plasma membrane contact (Knutton and Pasternak, 1979). Other methods of fusion have been explored, including the application of electric field pulses to induce fusion of cells (Vienken and Zimmerman, 1985).

3. Advantages of Monoclonal Antibodies

The success of monoclonal antibodies has arisen out of their many advantages over polyclonal antisera. Large amounts of monoclonal antibodies can be generated very easily by batch culture or ascites production (Yelton et al., 1981). To generate large amounts of polyclonal antisera, many animals are needed and/or re-immunization is required. Because monoclonal antibodies are a single antibody species, whose properties remain constant, they can be used as a standard reagent in immunoassays. This is not the case with polyclonal antisera since the properties may change from one

immunization to the next. Also, the supply of polyclonal antibodies ends when the animal dies. Hybridomas can be stored in liquid nitrogen indefinitely, thus providing an endless supply of monoclonal antibodies (Eshhar, 1985). Another advantage is their manipulability, since hybridoma cell lines can often be mutated to produce antibodies not found in nature (Kohler, 1986). Although cross-reactions are not completely eliminated with the use of monoclonal antibodies, they are minimized since a single antibody species is utilized. Lastly because one is selecting a single antibody species from a whole population, the antigen preparation does not have to be pure provided the assay method can distinguish between antibodies to the antigen and antibodies to the impurities (Bankert et al., 1984).

4. Applications to Food Science

While monoclonal antibodies have been used extensively in the medical field, uses related to food science have been limited. Currently their use is expanding, particularly in areas where immunoassays have already been applied.

Skerritt (1985) isolated two antibody clones which bound specifically to certain low-mobility prolamins in wheat, rye, barley and oats. These proteins were stable to heating so they could possibly be useful in an assay for gluten in cooked or processed foods. In a previous paper (Skerritt et al., 1984) used similar monoclonal antibodies to examine the homology between the storage proteins of different cereal grains, since each monoclonal could be highly specific for a

given amino acid sequence.

Kaminogawa et al. (1987) produced a monoclonal antibody which could bind both native and unfolded B-lactoglobulin. These researchers suggested that this monoclonal antibody would be useful in identifying that part of the molecule which stimulates allergies. In addition, the antibody could be used to study the unfolding and refolding of the protein and its surface structure.

The majority of work with monoclonals in food science is with food pathogens and their toxins. In 1983, Robison et al. reported on a monoclonal antibody called MOPC 467 which was able to specifically detect Salmonella bacteria in mixed culture and at the same time did not cross react with other enteric bacteria. This antibody was found to be specific for a flagellar determinant. Butman et al. (1988) raised genus-specific monoclonal antibodies for Listeria which showed no cross-reactions with a panel of non-Listeria species, including those to which it is antigenically related. The researchers identified the antigen as a heat-stable protein with a molecular weight of 30,000 - 38,000 daltons. Farber and Speirs (1987) raised monoclonal antibodies against a flagellar antigen common to Listeria species. These also did not cross react with any of the 30 non-Listeria strains tested. These researchers successfully demonstrated the use of these monoclonal antibodies in an immunoassay of milk and cheese samples.

Monoclonal antibodies have also been derived for

bacterial toxins. One example of this application is work by Shone et al. (1985) who produced a monoclonal antibody specific for Type A neurotoxin of Clostridium botulinum. When this monoclonal antibody was used in an immunoassay of salmon and corned beef samples, detection limits were close to that of the mouse bioassay which is commonly used for detection of this toxin. Other examples include work by Wnek et al. (1985) who derived a monoclonal antibody specific for Clostridium perfringens Type A enterotoxin and Edwin et al. (1984) who produced monoclonal antibodies specific for staphylococcal enterotoxin A.

C. Escherichia coli

1. E. coli and the Incidence of Infantile Diarrheal Disease

While the incidence of infantile diarrhea in developed countries is quite low, it is, not surprisingly high in underdeveloped countries where hygiene standards are poor and the children are most often malnourished. In developing countries, it is reported that diarrheal diseases cause 3 to 5 million child deaths per year (WHO, 1987a) and is estimated to be responsible for 25% of all deaths in children under 5 years of age (WHO, 1987b). These numbers are expected to decline in coming years due to the implementation of a Diarrheal Disease Control Program which makes use of oral rehydration therapy and vaccines specific to some causative agents. Due to problems in training and logistics, the success of this program has been limited to only a few areas and therefore morbidity from diarrhea remains high.

The major etiological agents of diarrhea in developing countries include Shigella dysenteriae and S.flexneri, Vibrio cholera, Salmonella typhi, rotavirus and others (WHO, 1987b). In cases where the causative organism was identified, the different strains of E.coli are together the most common cause of acute diarrheal illness (Black et al.,1981; Guerrant et al., 1983).

2. Types of E.coli

There are presently four known classes of E.coli that cause diarrhea. They are: (1) enterohemorrhagic; (2) enteroinvasive; (3) enterotoxigenic and (4) enteropathogenic E.coli. In addition to clinical symptoms, epidemiology and O - antigen serogroups, each class can be distinguished by its pathogenic mechanism (Rennels and Levine, 1986).

Enterohemorrhagic E.coli (EHEC) exerts its pathogenicity through a cytotoxin which appears to be identical to Shiga toxin (O'Brien et al., 1983). Enteroinvasive E.coli (EIEC) invades the epithelial cells of the intestine and causes histological damage leading to diarrheal symptoms (Editorial, 1983). Enterotoxigenic E.coli (ETEC) is best known as the cause of traveller's diarrhea, but it is also a major cause of infantile diarrhea in developing countries. These E.coli are characterized by the presence of adhesion organelles by which the bacteria adhere to the intestine in addition to enterotoxin production. The toxins produced are either heat labile (LT) or heat-stable (ST) (Rennels and Levine, 1986).

Enteropathogenic E.coli (EPEC) were the first class of

E.coli to be recognized, although their exact mode of pathogenicity remains unknown. The World Health Organization E.coli Centre recognizes 14 O serogroups as enteropathogenic including O20, O26, O86, O142, O55, O111, O114, O119, O125, O126, O127, O128, O44 and O158 (Back et al., 1980). It is largely thought that these bacteria cause diarrhea without tissue damage and without producing toxin (Moriarty and Turnberg, 1986).

3. Mode of Pathogenicity of EPEC

Considerable research has gone into the search for the mode of pathogenicity of enteropathogenic E.coli.

During examination of EPEC infection in intestinal biopsies of animal models and ill infants, it was found that there was a histopathogic lesion in the small bowel (Rennels and Levine, 1986). EPEC have been shown to adhere to the intestinal mucosa and produce an "attaching and effacing" (AE) lesion in the brush border microvillous membrane. This lesion involves localized destruction of microvilli and attachment of the bacteria to the apical enterocyte membrane (Knutton et al., 1989). It is possible that this damage leads to a decrease in absorptive surface area, resulting in diarrhea (Rothbaum et al., 1983). This lesion has been shown to occur in infections of all serotypes of EPEC tested so far (Rennels and Levine, 1986).

It has also been shown that of all the classic EPEC strains, 80% are able to adhere to HEp-2 (human epithelial) cells in tissue culture in the presence of D-mannose. This

characteristic is not seen in other strains of E.coli (Cravioto et al., 1979). The ability to adhere to HEp-2 cells in vitro has been shown to be plasmid-mediated. The genes encoding this adhesiveness are located on a 50-70 megadalton plasmid (Levine et al., 1985). This plasmid-mediated adhesion has been termed the EPEC adherence factor or EAF (Rennels and Levine, 1986).

Studies in Peru showed that HEp-2 adhesiveness is more commonly found in those EPEC serotypes implicated in epidemic diarrhea (Class I strains) rather than those that cause infrequent outbreaks (Class II strains) (Nataro et al., 1985). Levine et al. (1985) reported that while the EAF plasmid is necessary for full expression of pathogenicity of Class I strains, Class II strains do not have this plasmid yet can still cause diarrhea. In addition, Class II strains either do not adhere to HEp-2 cells in vitro or show diffuse adherence (Levine, 1987). Therefore another mechanism of pathogenicity must occur.

Levine et al. (1985) also showed that volunteers given a strain of E.coli containing EAF developed antibodies to a 94 kilodalton plasmid-associated outer membrane protein that is found in other Class I EPEC but not in enterotoxogenic E.coli. These researchers suggested that this protein might play a role in adherence of these bacteria in the gut.

Despite these observations, it has been postulated that this adherence in itself is not what causes diarrheal disease (Rothbaum et al., 1983). Rothbaum et al. (1983) suggested

that at least one serogroup of EPEC may produce a cytotoxin that affects protein synthesis, eventually resulting in cell death and loss. O'Brien et al. (1982) and Cleary et al. (1985) found that many EPEC serotypes produce a toxin similar to that of Shigella dysenteriae Type 1 (Shiga). Cleary et al. (1985) found that this Shiga-like cytotoxin was detected more often and in larger amounts in EPEC than in other fecal E.coli. Therefore, this toxin may play a role in the pathogenisis of EPEC-related gastroenteritis.

4. Detection of EPEC

The study of bacterial enteric pathogens typically involves first isolating them on selective media from the total bacterial population present in the feces, then testing individual colonies in biochemical reactions followed by agglutination in specific antisera (Echeverria et al., 1985). Until recently, this was the only way researchers could identify EPEC strains. This method is expensive, labourious and somewhat unreliable (Robins-Browne, 1987).

Most recently Nataro et al. (1985) developed a DNA hybridization probe from a 1-kilobase segment of the EAF plasmid. They found this probe to be highly sensitive and specific in detecting EPEC strains that exhibit HEp-2 localized adhesiveness. However, this probe is incapable of detecting Class II EPEC which either do not adhere to HEp-2 cells or adhere diffusely. Therefore, the development of a more practical technique such as using antisera to EPEC virulence-associated antigens is desired. Such a technique

might involve the use of monoclonal antibodies.

Numerous researchers have used monoclonal antibodies in their study of the E.coli bacteria. Monoclonal antibodies have been successfully raised against the heat-lable (LT) and heat stable (ST) enterotoxins of enterotoxigenic E.coli (Svennerholm et al., 1986) in addition to the colonization factor antigens (CFA) which allow some strains of ETEC to colonize the intestine (Lopez-vidal et al., 1988). These monoclonal antisera can be used to detect the presence of ETEC strains. However, a practical technique for the detection of EPEC strains using monoclonal antisera remains to be developed. Such a technique would be useful to both researchers and diagnosticians.

D. Immunoassays

1. Introduction

Classical methods for the analysis of foods often involve lengthy extraction procedures using organic solvents and/or chromatographic techniques. The use of an immunoassay offers a simple and economical alternative with often improved sensitivity over conventional analytical methods.

Immunoassays are based on the ability of antibodies to bind to a specific antigen (Pesce et al., 1978). Chemical measurements based on this interaction have been carried out in a clinical setting for the past 20 years. However, it is just recently that this methodology has begun to have an impact in food analysis due to an increased demand for analytical methods with lower detection limits. This is, in

part, due to increased concern over food safety and regulation.

The development of methods which use labelled antibodies or antigens has resulted in assays with high levels of sensitivity and specificity. Labels used in immunoassays have included fluorescent labels in fluorescence immunoassay (FIA) and isotopes in radioimmunoassay (RIA) (Voller et al., 1978). However, these labels have limitations. FIA is time consuming and not easily automated while RIA depends on isotopes as reagents which have a short shelf life and are under regulatory control. In addition, expensive equipment and skilled personnel are required (Monroe, 1984).

To date, use of antigens or antibodies labelled with enzymes such as horse radish peroxidase or alkaline phosphatase has proven to be most successful in immunoassays (Voller, 1980). Enzyme Immunoassays (EIA) are comparable to FIA and RIA in sensitivity, often detecting substances in the nanogram to picagram range. In addition, not only can EIAs often be completed in only a few hours, but also minimal equipment and training is required. Also, enzymes have a longer shelf-life than fluorescent labels or isotopes (Monroe, 1984).

2. Enzyme-Linked Immunosorbent Assay

In 1971, Engvall and Perlmann described an EIA in which the analyte to be detected binds either to the antigen or antibody which is coated on a solid surface. Molecules that do not bind are washed away from the solid surface and the

adsorbed enzyme conjugate measured by chemical reaction with substrate (Monroe, 1984). Engvall and Perlmann (1971) called this immunoassay an Enzyme-Linked Immunosorbent Assay or ELISA. This assay could be used to detect either antibodies or antigens (Engvall and Carlsson, 1976).

The solid phase in an ELISA can be in the form of particles of cellulose, polyacrylamide or agarose, or it can be preformed into discs, tubes, or beads (Voller, 1980). The most popular form of ELISA involves passive adsorption of the reactant to polystyrene microplates (Voller, 1980).

ELISAs can be broken down into three types: competitive, sandwich and indirect. In a competitive ELISA, the test sample containing the antigen is mixed with a known concentration of enzyme-labelled antigen. Both then compete for a limited number of binding sites on antibodies adsorbed on the microplate. In the sandwich ELISA, the antigen being assayed is held between antibody adsorbed on the microplate and a second antibody which contains the enzyme label. The indirect ELISA is generally used for antibody detection. In this assay the antigen is adsorbed on the microplate. Sample containing antibody is then added, followed by enzyme-labelled antiglobulin (Monroe, 1984).

3. Applications of ELISA to Food Science

ELISA has been used successfully for both qualitative and quantitative detection of food analytes, contaminants and disease agents.

Saunders and Clinard (1984) developed an ELISA that could detect Trichina spiralis in 30-60 minutes. Ruitenberg et al. (1983) demonstrated that ELISA can detect as little as 1 larva/100g of muscle versus 1 larva/g by digestion methods and 3 larva/g by trichonoscopy.

ELISA has also been used to detect molds and mycotoxins in foods. Notermans et al. (1986) isolated a heat stable mold specific antigen from a mold culture. This antigen was specific to molds and was produced by both Penicillium and Aspergillus species which are the predominant molds in foods. Using this antigen, these researchers developed an ELISA for the detection of these molds in foods. With respect to mycotoxins, as little as 50 pg of aflatoxin B_1 can be detected in peanut butter using a monoclonal antibody in an ELISA (Ram et al., 1986).

Patterson and Jones (1983) were able to distinguish meat of different species using an ELISA which detected different serum albumins. A similar ELISA was used to detect soya protein among meat proteins (Crimes et al., 1984).

The majority of applications of ELISA to food science have been for the detection of bacteria and their toxins in foods. Litton Biotechnics sells a "Bioenzabead Diagnostic Kit" which is an ELISA for the detection of Salmonella in foods. This kit provides a time saving of 1 to 2 days over official culture methods. The kit employs a monoclonal antibody which is capable of detecting 94% of all Salmonella strains (Eckner et al., 1987).

One of the most promising areas where ELISA might be applied is in the detection of Listeria. As was mentioned previously in this paper, Farber and Speirs (1987) developed a monoclonal antibody against Listeria which could have potential use in an ELISA.

Bacterial toxins have also been detected using ELISA, for example Staphylococcus enterotoxin A (Saunders and Bartlett, 1977) and E.coli enterotoxins (Svennerholm et al., 1986).

ELISA has also had application in the detection of enzymes. Vaag (1985) described the use of an ELISA to control the addition of enzyme preparations such as papain and amyloglucosidase to beer. In addition, Stepaniak et al. (1987) used an ELISA to monitor the production of heat stable proteinases and lipase from Pseudomonas in order to evaluate the quality of cold stored milk. Compared with agar diffusion methods for proteolytic and lipolytic activity, detection limits for purified proteinase P1 were 0.25 ng/ml and 120 ng/m of milk by ELISA and agar diffusion respectively. Detection limits for purified lipase P1 were 0.25 ng/ml of milk by ELISA and 1900 ng/ml of milk by agar diffusion.

Another potential application of the detection of enzymes by ELISA was reported by McCannel (1988) and Yoshioka (1988). They suggested that an ELISA could be used to detect the enzyme ß-N-acetylglucosaminidase in fish muscle. They suggested that this assay could be used as a means of

differentiating between fresh fish and fish that had been frozen then thawed.

4. Use of ELISA to Detect ß-N-acetylglucosaminidase (NAGase)

Physical, chemical and organoleptic changes in fish are of great commercial importance (Quaranta and Perez, 1983). In Europe, it has been reported that sea-frozen fillets are often thawed in the fish shops and sold as fresh fillets (Rehbein, 1979). Because it is not always visually apparent whether fish has been frozen, a detection method based on physical or chemical changes would be useful in quality control.

A wide variety of methods have been proposed for the measurement of quality changes in fish during frozen storage. In addition to sensory attributes, they include assays to measure changes in the nature and composition of free fatty acids, production of carbonyls from lipids and development of rancidity, measurement of extractable proteins, trimethylamine content and enzyme properties (Quaranta and Perez). However, use of any one of these methods may not necessarily be indicative of the freezing process alone.

Despite the extensive studies done on the quality of frozen fish, attempts to distinguish between frozen/thawed and fresh fish fillets have been largely unsuccessful.

Yoshioka and Kitamikado (1988) showed that erythrocytes in fish were destroyed when frozen and that this destruction could be detected by microscopic examination of blood samples. This method was applied to differentiate between

fresh and frozen/thawed fish. Yoshioka (1983a) also used a hematocrit value as an index of blood cell destruction for the same purpose. The same researcher distinguished frozen/thawed fish from fresh fish by examination of the medulla of the lens of the fish, since it was found that it became opaque during frozen storage (Yoshioka, 1983b). These methods were found to be time-consuming and impractical.

Some research has involved the use of enzymes for detection of frozen/thawed fish. The release of the mitochondrial form of glutamate-oxaloacetate-transaminase (E.C. 2.6.1.1) by freezing and thawing was used successfully for the detection of frozen/thawed beef and pork (Vandekerckhove et al., 1972) yet was found to be inadequate for fish fillets since mitochondria were also found to be destroyed by autolysis during iced storage of fresh fillets (Hamm and Masic, 1971). Another disadvantage of using mitochondrial enzymes as an indicator of freezing is the possible existence of isozymes in the cytoplasm (Rehbein et al., 1978). For this reason, electrophoresis or some other separation method is required.

Subsequently, Rehbein et al. (1978) and Rehbein (1979) found the use of lysosomal enzymes to be more promising. Fish muscle contains various lysosomal enzymes, many of which are released during freezing and subsequent thawing of a fillet or the isolated lysosomal fraction. These researchers found β-N-acetylglucosaminidase (NAGase) (E.C. 3.2.1.30) and α-glucosidase (E.C. 3.2.1.20) to show the most promise.

Using a spectrophotometric method to determine enzyme activity, they found that the press juice/extract activity ratio increased 6-9 times for α-glucosidase and 3-5 times for NAGase during freezing and thawing of fillets from cod, saith, red fish and haddock.

Yoshioka (1988) and McCannel (1988) proposed the use of an ELISA for the detection of NAGase as a means of distinguishing between fresh and frozen/thawed fish fillets. They successfully isolated anti-NAGase immunoglobulins from the eggs of chickens immunized with commercially prepared bovine kidney NAGase. Using this antibody, an indirect ELISA was developed to measure the NAGase concentration in the press juice and extract of both fresh and frozen fish fillets. The results obtained were ambiguous. The higher the dilution of sample applied, the higher the concentration of NAGase (based on a standard curve with bovine kidney NAGase). McCannel (1988) suggested that this may have been due to the presence of other substances in the juice and extract which interfered with NAGase binding to the plate. Patterson and Jones (1985) reported a similar phenomenon in their ELISA for species identification of meat. They offered a similar explanation.

In addition to these results, Yoshioka (1988) found that for salmon, samples frozen for one week showed consistently lower NAGase concentrations than the fresh samples. However, examination of the same samples for enzyme activity showed increased NAGase activity in frozen samples. Similar results

were found when the same tests were performed on fresh and frozen bovine kidney samples. McCannel (1988) suggested that this may be due to a possible change in the antigenic structure of the NAGase as a result of freezing.

MATERIALS AND METHODS

PART 1: MONOCLONAL ANTIBODY (Mab) STUDY

A. Hybridoma Production

The protocol used for hybridoma production is that outlined by Kannangara et al. (1989) with modifications (Figure 30). All aspects of hybridoma production were carried out under sterile conditions.

1. Outer Membrane Preparation

Cultures of E.coli O142:K86:H6 (ATCC 23985) were donated by Dr. B. Skura. A loopful of cells was transfered to each of 5 individual 250 ml flasks containing 150 ml of tryptic soy broth (TSB) (Difco Labs., Detroit, MI) and incubated in a Controlled Environment Incubator Shaker (NB Scientific Co. Inc., Edison, NJ) at 80 rpm and 37^{o}C for 24 hr. Cells were harvested by centrifugation at 12,000 X g for 10 min at 5^{o}C. Cells harvested from approximately 200 ml of culture were resuspended in 10 ml of breaking buffer (10 mM Tris-HCl, 20% sucrose (w/v), 5 mM magnesium chloride ($MgCl_2$)) and disrupted using a French Press (Loomis Engineering and Mfg. Co., Caldwell, NJ) at 10,000 - 12,000 psi. Unbroken cells were removed by centrifugation at 5000 X g for 10 min at 5^{o}C. The broken cells were loaded into SW-41 tubes (Beckman Instruments Inc., Toronto, ON) with the following sucrose step gradient: 2 ml of 70% sucrose (w/v) overlayed with 7 ml of 60% sucrose (w/v). The tubes were spun at 200,000 X g for 3 hr at 5^{o}C in a Beckman L8-80 ultracentrifuge (Beckman

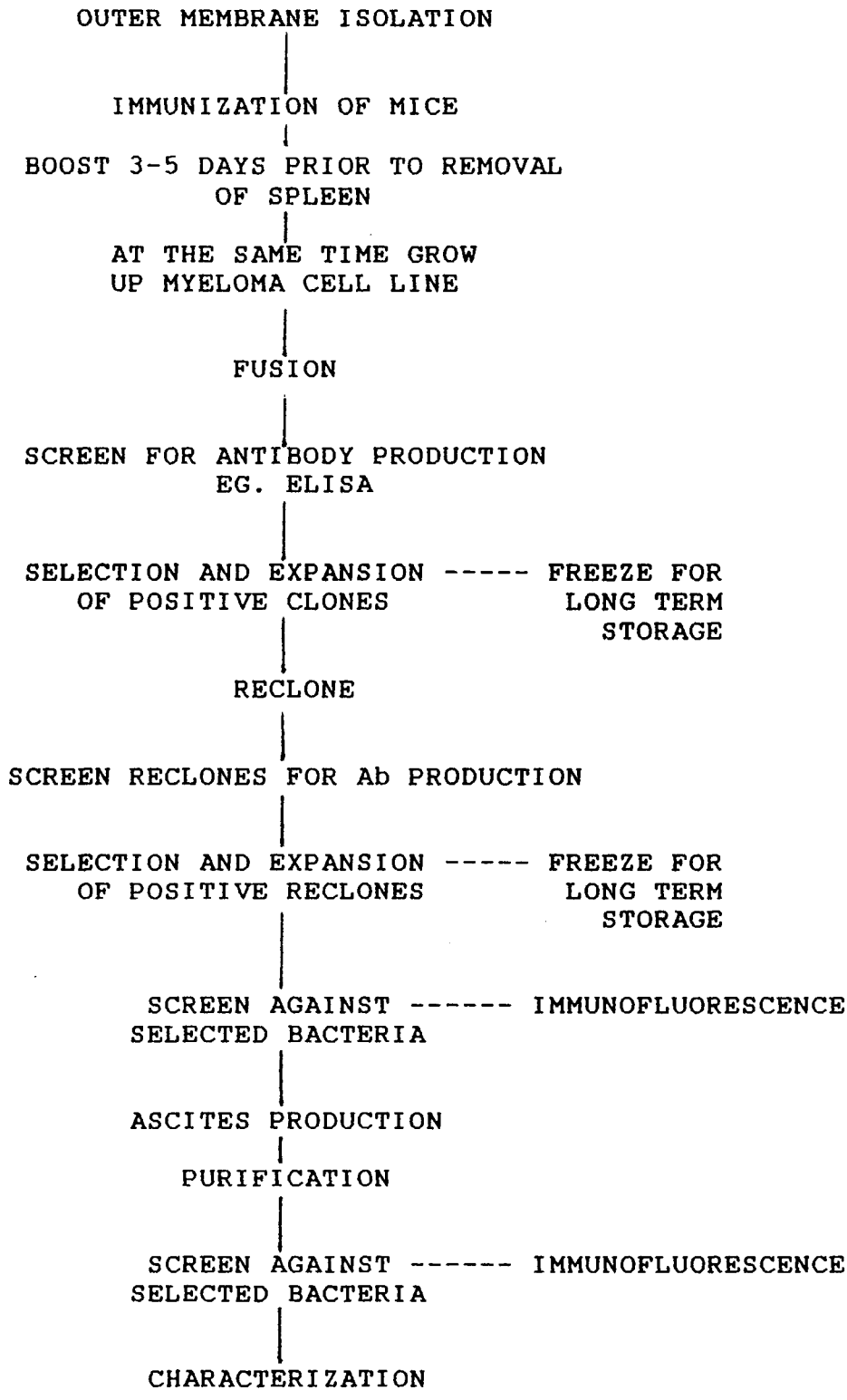

Figure 3: Protocol for Mab production.

Instruments Inc., Toronto, ON). The layer at the boundary of the 60% and 70% sucrose gradient was harvested with a pasteur pipette. The resulting sample of approximately 6 ml was split between two Ti-70 tubes (Beckman Instruments Inc., Toronto, ON) and the remainder of the space filled with 0.01 M phosphate buffered saline (PBS), pH 7.4. The tubes were spun at 265,000 X g for 2.5 hr at 5^{o}C. Each pellet was redissolved in 200 μl of PBS and stored at 4^{o}C.

2. Immunization of Mice

Four 3 month old balb/c mice (Agriculture Canada Research Station, Vancouver, BC) were injected subcutaneously with 50 μg of the outer membrane preparation mixed 1:1 with Freund's incomplete adjuvant (Difco Labs., Detroit, MI) using a 22G1/2 needle. The mice were then rested for one month. Four days prior to fusion the mice were boosted intraperitoneally with 20 μg of the outer membrane preparation without any adjuvant.

3. Growth of Myeloma Cell Line

Five days prior to fusion, one vial of Fox-NY myeloma cells (Hyclone Labs., Logan, UT) was removed from a liquid nitrogen freezer (-150^{o}C) and allowed to thaw by holding the vial under running lukewarm tap water. The cells were resuspended in 10 ml of Dulbecco's Modified Eagle's medium (DME) (Gibco/BRL, Burlington, ON) and centrifuged at 800 X g for 10 min at room temperature (RT). The pellet was resuspended in 10 ml DME containing 20% fetal calf serum

(FCS) (Hyclone Labs., Logan, UT) in a sterile Petri plate. This was incubated at 37°C in an incubator with an atmosphere of 10% carbon dioxide (CO_2). When the cells covered approximately 50 - 70% of the surface of the plate, the culture was split into two plates and fresh medium added. This was repeated until six or more plates containing cells covering 50 - 70% of the plate were obtained.

4. Fusion

Resuspended cells from six Petri plates of myeloma cells were centrifuged at 800 X g for 10 min at RT. Cells were harvested and washed by resuspending the pellet in 10 ml DME followed by centrifugation.

Five days following boosting, one of the immunized mice was sacrificed and the spleen removed. The spleen was placed in a sterile Petri plate containing 10 ml DME and a one inch square of sterile guaze placed over it. The spleen was minced through the gauze using the end of a sterile syringe plunger. Spleen cells were harvested by centrifugation at 800 X g for 10 min at RT. The pellet was resuspended in 10 ml DME and transfered to a clean test-tube. The centrifugation step was repeated.

The pellets of myeloma and spleen cells were resuspended together in 10 ml DME followed by centrifugation at 800 X g for 10 min at RT. The supernatant was aspirated off and the bottom of the test-tube containing the pellet was immersed in a 37°C water bath. While immersed, 1 ml of fusogen (50% polyethylene glycol 4000 (w/v) (Sigma, St. Louis, MO), 10%

dimethyl sulfoxide (DMSO) in DME) was added slowly over 1 min while gently stirring with a pipette. One millilitre of DME was then added slowly over 1 min while stirring, followed by 2 ml of DME over 1 min and an additional 6 ml over 3 min. This mixture was centrifuged at 800 X g for 10 min at RT and the pellet resuspended in 5 ml of AHAT medium (DME containing 20% FCS and 5 ul of a stock solution containing 2.58 mg/ml adenine (Sigma, St. Louis, MO), 1.36 mg/ml hypoxanthine (Sigma, St. Louis, MO), 0.176 mg/ml aminopterin (Sigma, St. Louis, MO) and 0.776 mg/ml thymidine (Sigma, St. Louis, MO)).

Two 4 - 6 week balb/c mice, which were not immunized, were sacrificed and the thymuses removed. The organs were minced and the thymocytes collected using the same procedure as for the spleen cells. The thymocytes were mixed with the fused cell mixture and diluted to 50 ml with DME. This mixture was plated out at 100 ul per well into 5 sterile Nunclon 96 microwell plates (Gibco/BRL, Burlington, ON). The plates were incubated at 37^{o}C in 10% CO_2. After 3 - 4 days 100 μl of AHT medium (AHAT medium without aminopterin) was added to each well. After 7 days 180 μl of medium was removed from each well and replaced with fresh AHT medium. Three days later, hybridoma clones were tested for antibody production. Selected positives were expanded and recloned.

B. Expansion

Cells in wells containing selected positive hybridoma clones were resuspended in 1 ml of AHT media in a Nunclon 24 well tissue culture plate (Gibco/BRL, Burlington, ON). The

plates were incubated at 37°C in 10% CO_2. Three days later the wells were retested for antibody production. Cells from selected positive wells were expanded into 10 ml of DME containing 20% FCS and incubated as above. Once cells reached a high cell density they were expanded to a 10 times larger volume of medium.

C. Recloning

Recloning was performed to ensure homogeneity of hybridoma clones. Thymocytes from two young balb/c mice were obtained using the method described in the fusion protocol. The pellet was resuspended in 10% DME and the suspension added to approximately 60 ml of HAT media. The number of cells in the culture of clones to be recloned was counted using a heaemocytometer (CanLab, Richmond, BC) Based on the number obtained, the original stock of cells was diluted with AHAT media containing the thymocytes such that 50, 20, and 5 cells per ml were obtained. The dilutions were plated out at 100 µl per well in a Nunclon microwell plate (32 wells per clone per dilution). The plates were incubated at 37°C in 10% CO_2. Reclones were allowed to grow until they were large enough to score (observed visually using a light microscope with a magnification of 200X - 400X). Those wells containing only one clone were recorded and tested for antibody production. Selected positives were expanded as described previously.

D. Freezing for Long Term Storage

Selected hybridoma cultures, both before and after recloning, were frozen for long term storage. Cultures in two 10 ml Petri plates with a high cell density and exhibiting logarithmic growth were centrifuged at 800 X g for 10 min at RT. The pellet was resuspended in 3 - 5 ml of a cryoprotectant solution containing 20% FCS and 10% DMSO in DME. One millilitre volumes were transfered to Nunc Freezer vials (Gibco/BRL, Burlington, ON) and placed in an insulated cardboard box (approximately 30 cm^3 lined with 2.5 cm of styrofoam insulation). This was placed in a -80^{o}C freezer for 24 hr then transfered to liquid nitrogen.

E. Ascites Production

Large scale production of monoclonal antibodies was accomplished through ascites production in mice. Eight 3 - 4 month old balb/c mice were injected intraperitoneally with 0.5 ml pristane (Sigma, St. Louis, MO) using a 21G1 needle. One week later, 2 Petri plates of hybridoma cultures with a high cell density in log phase of growth were centrifuged at 800 X g for 10 min at RT and the pellet resuspended in 0.5 ml DME. This was injected intraperitoneally using a 22G1/2 needle into each pristane treated mouse (2 mice per hybridoma clone). Seven days later the ascites fluid was collected. This was done by inserting an 18G1/2 needle into the swollen abdomen of the mice and holding the mice over a 10 ml test-tube until the fluid flow stopped. The fluid collected was centrifuged at 800 X g for 15 min at RT. The supernatant was

divided into 1 ml aliquots and frozen at $-20^{o}C$.

F. Purification of Ascites Fluid

1. Gel Filtration Chromatography

Purification of ascites fluid by gel filtration chromatography was performed on a column packed with Sephacryl S-300 SF (Pharmacia, Dorval, PQ), using a 0.1 M Tris-HCl buffer, pH 8, containing 0.5 M sodium chloride (NaCl) as the eluting agent. Column dimensions were 1.8 X 34 cm. A solution of 10 mg/ml Blue Dextran 2000 (Pharmacia, Dorval, PQ) was applied in order to check the column packing and determine the void volume (V_o). A 1 ml sample of ascites fluid filtered through a Millex-GS 0.22 um filter unit (Millipore Corp., Bedford, MA) was applied under eluent flow. Eluent flow rate was 2 ml h^{-1} from top to bottom. Two millilitre fractions were collected and the protein concentration monitored by measuring the absorbance at 280 nm using a Shimadzu UV-Visible Recording Spectrophotometer (Shimadzu Corp., Kyoto, Japan). Immunoreactivity of selected fractions was monitored by indirect ELISA. Collected fractions were concentrated to 1 ml using a Centriprep-30 concentrator (Amicon, Danvers, MA) and sodium azide (NaN_3) added to 0.02%.

2. Ion-Exchange Chromatography

Purification of ascites fluid by ion-exchange chromatography was performed on a 10 ml plastic syringe (Becton-Dickson, Rutherford, NJ) packed with DEAE-Sephacel

(Sigma, St. Louis, MO) using a 10 mM Tris-HCl buffer, pH 8.0 as the eluting agent. Column dimensions were 1.8 X 7 cm. To apply the samples, eluent flow was stopped and the buffer allowed to drain to the top of the column bed. One millilitre of filtered ascites fluid was applied to the top of the column bed using a pasteur pipette and eluent flow resumed. Eluent flow rate was 5 ml h^{-1} from top to bottom. One millilitre fractions were collected and the protein concentration and immunoreactivity monitored as described in gel filtration. Collected fractions were concentrated to 1 ml and NaN_3 added to a concentration of 0.02%.

A linear elution gradient was created by using two cylinders of like dimensions connected with tubing. The first cylinder contained 20 ml of the eluting buffer while the second cylinder contained an equal volume of 1 M NaCl. During eluent flow the two were mixed with a magnetic stir bar as gravity caused the salt solution in the second cylinder to move into the first.

G. Detection of Antibody Activity Towards E. coli

1. Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA described by Kannangara et al. (1989) was used to screen hybridoma cultures for antibody production. Unless otherwise stated, volumes of samples and reagents added were 100 µl per well. A Linbro/Titertek EIA microtitration plate (Flow Laboratories, McClean, VA) was coated with 10 µg/ml of outer membrane preparation in PBS. Plates were incubated either for 1 hr at 37°C or overnight at

4°C. Following incubation the coating solution was removed by shaking the plate contents into a sink. Blocking solution (2% Blotto: 10% skimmilk powder in deionized distilled water containing 0.02% NaN_3 in PBS) (PBS Blotto) was added (200 µl per well). For each sample well required, a second well containing no coating was prepared as a control. Incubation was carried out for 30 min at 37°C and the blocking solution removed. Fluid from each hybridoma culture was added and the plates incubated for 1 hr at 37°C. Following washing with tap water, rabbit anti-mouse alkaline phosphatase (ALP) conjugate (Bio/Can Scientific Inc., Mississauga, ON) diluted 1/3000 with PBS Blotto was added and the plates incubated for 1 hr at 37°C. After a final wash, 0.05% p-nitrophenyl phosphate (Sigma, St. Louis, MO) in 10% diethanolamine buffer, pH 9.8, containing 0.01% $MgCl_2$ and 0.02% NaN_3 was added and the plates incubated until a colour reaction was observed. The absorbance of each well at 405 nm was read using a Titertek Multiscan MCC ELISA reader (Flow Labs., McClean, VA). Control values were subtracted from sample values.

A similar ELISA was used to monitor the immunoreactivity of the gel filtration and ion-exchange fractions. Instead of adding hybridoma culture fluid to the wells, 100 µl of each column fraction was added.

2. Immunofluorescence

An immunofluorescence assay was performed to test the monoclonal antibodies produced for cross-reactivity with

other bacteria. Cultures of the following bacteria were obtained from Mr. J. Jessop of the B.C. Provincial Health Laboratory (Enterics): 5 serotypes of non-enteropathogenic E.coli: O157:H7, O157:K88:H19 and three for which no serotype information was given (labelled #1,2 and 3); five serotypes of enteropathogenic E.coli: O128:K67, O55:K59, O44:K74, O112:K68, O18:K77, and thirteen other Enterobacteriaciae: Serratia marcescens, Citrobacter freundii, Enterobacter cloacae, Edwardsiella tarda, Klebsiella pneumoniae, Proteus mirabilis, Proteus morganii, Hafnia alvei, Escherichia fergosonii, Escherichia hermanii, Proteus rettgeri, Kluyvera ascorbita, and Alkalescens-dispar-I.

Slants of the above bacteria were prepared on tryptic soy agar (TSA) (Difco Labs., Detroit, MI) and incubated for 24 hrs at 37°C. To each slant 3 ml of deionized distilled water filtered through a Millex-GS 0.22 µm filter unit (Millipore Corp., Bedford, MA) were added. The slant was then agitated using a vortex mixer. The resulting cell suspension was added to a 50 ml centrifuge tube. Filtered water was added to approximately 40 ml followed by centrifugation at 15,000 X g for 20 min. The pellet was resuspended in 40 ml of the filtered water and the centrifugation step repeated. The pellet was resuspended in 2 ml filtered water and a dilution series of 10^{-1} to 10^{-4} prepared. Twenty microlitres of each dilution was placed in a well on a toxoplasmosis slide (Bellco Glass Inc., Vineland,

NJ) and allowed to air dry. Bacteria were heat fixed by passing the slides through a flame 4 times. Once the slides cooled, 20 µl of culture fluid from each hybridoma or 20 µl of purified Mab diluted 1/500 were placed in the wells. Negative controls contained no Mab solution. Slides were placed in a sealed plastic container and incubated for 30 min at 37°C then washed with distilled water and air dryed. Twenty microlitres of fluorescein-conjugated anti-mouse antibody conjugate (Bio/Can Scientific Inc., Mississauga, ON) diluted 1/1000 in PBS containing 5% Blotto were added, followed by a 30 min incubation at 37°C. After a final wash and air dry, one drop of mounting fluid (glycerol containing 10% PBS and 0.1% p-phenylenediamine (w/v)) was added to each well and the coverslips applied.

Slides were observed using a fluorescence microscope (model no. 64881, Carl Zeiss, W. Germany) equipped with a mercury vapour light source and set up for epi-illumination with a filter set for fluorescein fluorescence (excitation at 495 nm and emission at 520 nm). A 100X oil immersion Neofluar objective was used for observation.

H. SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using two methods.

1. Method A

The first method used was described by Weber and Osborn (1969). Ten percent acrylamide gels were prepared by mixing

the following: 4.8 ml of a 0.2 M sodium phosphate buffer, pH 7.0, containing 0.2% SDS (w/v) (Sigma, St. Louis, MO), 4.2 ml of a 22.2% acrylamide (w/v) (Sigma, St. Louis, MO) solution containing 0.6% bisacrylamide (w/v) (Sigma, St. Louis, MO), and 600 µl of a 1.2% ammonium persulfate (w/v) (Sigma, St. Louis, MO) solution. To initiate polymerization, 15 µl of N,N,N',N'-tetramethylethylenediamene (TEMED) (Bio-Rad, Richmond, CA) were added. Samples of eluted fractions from gel filtration and ion-exchange were mixed 1:1 with sample buffer containing 4% SDS in sodium phosphate buffer, containing 10% glycerol, 1% 2-mercaptoethanol and 0.01% malachite green (Fischer Scientific, Ottawa, ON). Ten microlitre samples were applied to the gels.

2. Method B

The second method used was described by Laemmli (1970). Twelve percent acrylamide separating gels were prepared by mixing the following: 3.35 ml distilled water, 2.5 ml of a 1.5 M Tris-HCl buffer, pH 8.0, 0.1 ml of a 10% SDS stock solution, 4 ml of a 30% stock solution of acrylamide/bis (Bio-Rad, Richmond, CA), and 25 µl of a 10% ammonium persulfate solution. Four percent stacking gels were prepared by mixing the following: 3.05 ml distilled water, 1.25 ml of a 0.5 M Tris-HCl buffer, pH 6.8, 50 µl of the SDS stock solution, 0.67 ml of the acrylamide/bis stock solution and 50 ul of the ammonium persulfate solution. Five microlitres of TEMED was added to each of the above mixtures to initiate polymerization. Samples were mixed 1:1 with

sample buffer containing 12.5% of a 0.5 M Tris-HCl buffer, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.01% bromophenol blue (Bio-Rad, Richmond, CA).

Gel mixtures from both methods were poured into a Bio-Rad slab type Vertical Gel Electrophoresis Unit (Bio-Rad Richmond, CA) with dimensions of 7.2 X 10 cm and a gel thickness of 0.75 mm. Samples used in both methods were placed in a boiling water bath for approximately 5 min prior to application to the gels. Standards used were Bio-Rad Molecular Weight Standards (Bio-Rad, Richmond, CA). Gels prepared by method A were run at 90 mA and gels prepared by method B were run at 25 mA until the tracking dye reached the stacking gel and then the amperage was increased to 50 mA. In both methods the gels were run until the tracking dye reached the bottom of the gels. Staining of gels was accomplished with 0.05% Coomassie Blue R250 (ICN, Cleveland, OH) and 0.05% Coomassie Blue G250 (Bio-Rad, Richmond, CA) in a solution of 30% methanol and 10% acetic acid for 1 - 2 hr. Destaining was done using a solution of 30% methanol and 10% acetic acid.

I. Characterization of Mabs

1. Isotyping of Mabs

Determination of the immunoglobulin isotype of the monoclonal antibodies was done using a Mouse Hybridoma Sub-Isotyping Kit (Behring diagnostics, La Jolla, CA). The procedure utilizes an ELISA similar to the one already described. Plates were coated with a 1/1000 dilution of goat

anti-mouse Ig and incubated for 1 hr at 37°C. After removal of the plate contents, blocking solution was added and the plates incubated for another 30 min. Plate contents were removed and monoclonal antibody samples, diluted 1/500 in PBS Blotto, were added. Control wells contained no antibody samples. Following incubation for 1 hr, plates were washed with tap water. For each antibody sample, the following rabbit anti-mouse immunoglobulins (Ig) diluted 1/1000 in PBS Blotto were added: IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. Plates were incubated for 1 hr and then washed with tap water. This was followed by addition of anti-mouse ALP conjugate and incubation for 1 hr. Enzyme substrate was added after a final wash. After incubation for approximately 1 hr, absorbance of the wells was read at 405 nm. Control values were subtracted from sample values.

2. Immunoblot Assay

An Immunoblot Assay of the monoclonal antibody samples was performed as described by Dunn (1986) with modifications. TSA slants of selected bacteria were prepared and a bacterial pellet obtained as was described in the immunofluorescence assay procedure. The pellet was resuspended in 2 ml of deionized distilled water. SDS-PAGE profiles were prepared using the method B described previously. For blots probed with Mabs 4D10 C1 and 2H4 H12, bacterial suspensions were prepared in sample buffer both with and without mercaptoethanol. Bacterial suspensions were mixed 1:1 with elecrophoresis sample buffer prior to application to the gel.

Seven microlitre samples were applied.

Gel profiles were transfered electrophoretically to an Immobilon-P (polyvinylidene difluoride) membrane (Millipore Corp., Bedford, MA) with a pore size of 0.45 μm using a TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA). The transfer buffer used contained 25 mM Tris, 192 mM glycine, pH 8.3, in 20% methanol (v/v). Blotting was carried out for 2 hr at 0.5 A. The temperature was kept cool by running cold water through the cooling coil. Following transfer, the gels were stained with a Coomassie Blue stain for 1 - 2 hr followed by destaining.

The membranes were placed in Petri plates and 10 ml of 5% Blotto added. Plates were incubated for 30 min at room temperature with stirring. The solution was decanted and 10 ml of monoclonal or polyclonal antibody sample, diluted 1/500 in PBS Blotto, were added. Incubation was repeated for 1 hr and the antibody solutions decanted. The membranes were washed by incubating them in 10 ml of PBS for 10 min. This was repeated 3 times with fresh PBS. After washing, 10 ml of rabbit anti-mouse Ig - ALP conjugate diluted 1/3000 or rabbit anti-chicken IgG - ALP conjugate (Sigma, St. Louis, MO) (for polyclonal samples) diluted 1/2000 with PBS Blotto was added. Membranes were incubated for an additional hour. The conjugate solutions were decanted and the washing step repeated with 50 mM Tris-HCl buffer, pH 8, containing 50 mM NaCl. Ten millilitres of substrate (0.33 mg/ml Napthol AS-MX phosphate disodium salt (Sigma, St. Louis, MO) and 3 mg/ml

Fast Red TR salt (Sigma, St. Louis, MO) in the above buffer) was added. Incubation was carried out in the dark. After approximately 30 min, the membranes were rinsed with PBS then air dryed.

J. Preparation of Polyclonal Antiserum to E. coli

1. E.coli Sample Preparation

E.coli O142:K86:H6 (ATCC 23895) cells which had been formalin treated (Shimizu et al., 1988a) and freeze-dried were a gift from Dr. S. Shimizu. Cells (2.2 mg) were dispersed in PBS previously sterilized by filtration through a Millex-GS 0.22 um filtration unit. This was mixed with an equal volume of adjuvant to yield approximately 10^8 cells/ml. An emulsion was formed by repeatedly drawing in and ejecting this mixture from a 5 ml syringe with a 20G3/2 needle.

2. Immunization of Chickens

Initial injections contained Freund's complete adjuvant, while subsequent injections contained Freund's incomplete adjuvant. Three laying White Leghorn hens obtained from the University of British Columbia Poultry Unit were given intramuscular injections of 0.25 ml of cell preparation to each of four sites (one in each breast and thigh) using a 26G3/8 needle. This injection was repeated approximately 2 weeks later.

3. Isolation and Purification of Chicken IgY

Eggs from the immunized hens were collected and stored at $4^{o}C$. The chicken IgY fraction was separated using a

method described by Polson et al. (1980) with modifications. Egg yolks were separated from the white and rinsed with distilled water. Yolks were punctured and the contents allowed to drain into a graduated cylinder. PBS (0.01M, pH 7.4) equalling 4 times the volume of the yolk was added followed by the addition of polyethylene glycol (PEG) (Sigma, St. Louis, MO) with a molecular weight of 8000 daltons to a final concentration of 3.5% (w/v). This mixture was stirred until the polymer dissolved. The mixture was then centrifuged at 14,500 X g for 20 min at 4^{o}C. The supernatant was decanted over cheesecloth to remove the lipid layer then filtered through Whatman No. 1 filter paper (Whatman Ltd., England). Twelve grams of PEG per 100 ml of filtrate was added. After stirring at RT for approximately 10 min, centrifugation was repeated. The supernatant was discarded and the precipitate dissolved in 200 ml of 0.02 M PBS, pH 7.2. Twelve percent PEG was added and the solution stirred until the PEG dissolved. Centrifugation was repeated and the precipitate collected and dissolved in 50 ml of a 25 mM potassium phosphate buffer, pH 8.0, per 100 ml of starting yolk.

Further purification of the chicken IgY was accomplished by ion-exchange chromatography. A 10 ml syringe was packed with DEAE-Sephacel equilibrated with 25 mM potassium phosphate buffer, pH 8. Twenty five millilitres of sample was applied under flow. The column was washed with 10 bed volumes of equilibrating buffer, then eluted with 250 mM

potassium phosphate buffer, pH 8. Flow rate was maintained at 0.75 ml min^{-1} from top to bottom. Six millilitre fractions were collected and absorbance at 280 nm monitored. Fractions from the peak eluted with the 250 mM phosphate buffer were pooled and concentrated by ammonium sulphate precipitation.

K. Ammonium Sulphate Precipitation

Ammonium sulphate was added to chicken anti-E.coli IgY fractions to give half saturation at RT (31.3 g/100 ml). This was stirred at RT for 30 min then centrifuged at 12,000 X g for 10 min at RT. The resulting precipitate was dissolved in 2 ml of PBS and dialysed against the same buffer (X2 changes) overnight at $4^{o}C$.

L. Protein Determination

Unless otherwise stated, protein determinations were done using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). A standard curve was prepared using either bovine serum albumin (Sigma, St. Louis, MO) or chicken IgG (Sigma, St. Louis, MO) ranging in concentration from 0.2 to 1.2 mg/ml in PBS. Blanks contained only buffer. To each tube, 100 ul of sample or standard and 5 ml of dye reagent (diluted five times with distilled water) was added. The mixture was vortexed and allowed to stand for 15 min. Absorbance at 595 nm was read and the protein concentrations of the samples calculated from the standard curve.

PART II: DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

A. Immunization Procedures

1. Immunization of Chickens

Preparation of immunogen and immunization of chickens was performed by Anne McCannel. ß-N-acetylglucosaminidase (NAGase) purified from bovine kidney (Sigma, St. Louis, MO) in a 3.2 M ammonium sulphate solution, pH 6, with a protein concentration of 5 mg/ml was mixed 1:1 with adjuvant in order to give a final protein concentration of 2.5 mg/ml.

Laying White Leghorn hens were obtained from the University of British Columbia Poultry Unit. Chickens were immunized using the procedure already described in PART I for preparation of anti-*E.coli* antiserum.

2. Immunization of Rabbits

Two New Zealand White rabbits were housed at the University of British Columbia Animal Care Centre. All injections and blood collections were done by Animal Care Centre personnel.

Sixty six microlitres of the NAGase solution used above were suspended in 1 ml of a saline solution (0.85% sodium chloride (w/v)). This was mixed 1:1 with Freund's complete adjuvant to give a final protein concentration of 165 ug/ml. To each rabbit, 0.1 ml of this preparation was injected subcutaneously into each of six sites using a 22G1/2 needle for a total of 100 ug protein. Three weeks later, a second injection was performed using an immunogen preparation

prepared in the following manner. Forty microlitres of NAGase solution were suspended in 200 µl of saline solution. This was mixed 1:1 with Freund's incomplete adjuvant to give a final protein concentration of 0.5 mg/ml. Each rabbit received 0.1 ml of this preparation intramuscularly into each of 2 sites for a total of 100 µg protein. Three weeks later the animals were bled by cardiac puncture. The blood was allowed to coagulate at RT, then centrifuged at 5000 X g for 30 min. The serum was collected and stored at 4°C with 0.02% NaN_3.

B. Immunoglobulin Preparation

1. Isolation and Purification of Chicken IgY

Eggs from immunized chickens were donated by Anne McCannel. Isolation and purification of chicken IgY from the egg yolks was carried out as described in PART I.

2. Isolation of Immunoglobulins from Rabbit Blood Serum

Isolation of the immunoglobulin fraction from the blood serum of immunized rabbits was carried out by ammonium sulphate precipitation as described by Garvey et al. (1977). The pH of a 25 ml aliquot of a saturated (RT) ammonium sulphate solution was adjusted to pH 7.8 by addition of 5 N sodium hydroxide. A 50 ml serum sample was added dropwise to this solution with constant stirring. The mixture was stirred for an additional 3 hrs at RT followed by centrifugation at 1400 X g for 30 min at RT. The resulting precipitate was dissolved in sufficient saline solution to

restore the volume of solution to that of the original serum sample. A second and third precipitation step was carried out. The final precipitate was dissolved in 5 ml of PBS and 0.02% NaN_3 added.

C. Preparation of Anti-NAGase - Alkaline Phosphatase Conjugates

Prepared anti-NAGase chicken IgY was conjugated to alkaline phosphatase (ALP) Type VII-S from bovine intestinal mucosa (Sigma, St. Louis, MO) using two different methods. A 2:1 molar ratio of ALP to chicken IgG was used in each method.

1. Glutaraldehyde Method.

The glutaraldehyde method used was described by Engvall and Perlmann (1972) and modified by Shimizu (1988b). Three hundred and sixty six microlitres of ALP solution were centrifuged at 1100 X g for 15 min at 4°C. The resulting precipitate was dissolved in 1.5 ml of PBS containing 1 mM $MgCl_2$ and dialysed against the same buffer (X2 changes) overnight at 4°C. Ten microlitres of a 25% glutaraldehyde solution (BDH Chemicals, Toronto, ON) were added and the mixture stirred for 50 min at RT. A 0.2 ml solution containing 3 mg previously prepared anti-NAGase chicken IgY in PBS was added and stirring continued for 75 min. The solution was cooled in an ice bath then applied to a Sephacryl S-300 SF column equilibrated with 50 mM Tris-HCl buffer, pH 8, containing 1 mM $MgCl_2$ and 0.1% NaN_3. Column dimensions were 1.3 X 50 cm. Eluent flow rate was 9

ml min^{-1} from top to bottom. One and a half millilitre fractions were collected and the protein concentration monitored as previously described. Fractions in the first eluted peak were pooled and stored at $4^{o}C$.

2. Periodate Oxidation Method

Anti-NAGase IgY was also conjugated to ALP by periodate oxidation. The method used was based on methods described by Williams (1984) and Tussen and Kurstak (1984) with modifications. Three hundred and sixty six microlitres of ALP solution were centrifuged at 1100 X g for 15 min at $4^{o}C$. The resulting precipitate was dissolved in 0.5 ml of a 0.3 M sodium carbonate solution and dialysed against the same overnight at $4^{o}C$ with two solution changes. To this solution, 0.5 ml of a 40 mM sodium periodate solution was added and the reaction allowed to proceed for 4 hr in the dark at RT with agitation. A 0.5 ml solution containing 3 mg anti-NAGase chicken IgY, previously dialysed overnight against a 0.01 M sodium carbonate buffer, pH 9.5, was added. The reaction was continued for 24 hr under the same conditions. To the reaction mixture, 40 µl of a 2 M ethanolamine solution, pH 9.5, was added, and the mixture dialysed overnight against PBS. Gel filtration chromatography was carried out as in the previous method.

D. Preparation of Press Juice and Fish Extracts from Fish Muscle

Fresh Sockeye salmon (not previously frozen) was obtained in chunks from a local seafood retail store. The

time the fish were stored on ice was not known. Samples were cut into 2.5 cm thick steaks and analysed on the day of collection and after one week of frozen storage. Steaks that were frozen were placed in individual freezer bags and then put in a cardboard box with dimensions of 10 X 30 cm. Storage was at -20^{o}C for one week. These samples were thawed overnight at 4^{o}C prior to analysis. Preparation of press juice and fish extract fractions from fish muscle was done according to Yoshioka (1988) with modifications. Fish muscle was ground using a Kitchen Aid meat grinder (Hobart Manufacturing Co., Troy, OH) by forcing the muscle through holes with a diameter of 5 mm. Five grams of the ground sample were placed in a filter-fuge tube (model 2427, International Equipment Co., Needham Heights, MA) and centrifuged at 9,600 X g for 30 min at 5^{o}C. Juice collected at the bottom of the filter-fuge tube was diluted to 2.5 ml with 1% NaCl and filtered using a Millex-HA 0.45 um filter unit (Millipore Corp., Bedford, MA). This was termed the press juice (PJ). The residue was weighed and a volume of 1% NaCl of 10 times the residue weight was added. This mixture was homogenized for 1 min at 12,000 rpm using an Ultra-Turrax Ika-Tron (Janke and Kunkel, Staufen, W. Germany) with an 18N probe. After equilibrating for 30 min at 4^{o}C , the mixture was centrifuged at 22,500 X g for 10 min at 5^{o}C. The supernatant was diluted to 20 ml with 1% NaCl then filtered through a Millex-HA 0.45 um filter unit. This was termed the fish extract (FE).

E. Enzyme-Linked Immunosorbent Assays

All ELISAs were performed on Immulon II microtitre plate (Dynatech Labs., Chantilly, VA). Bovine kidney NAGase used for animal injections was also used for coating plates and for standards. Unless otherwise stated, sample and reagent volumes added were 100 µl per well and incubation was for 1 hr at 37°C. Standard curves were derived using NAGase concentrations ranging from 0 to 20 µg/ml in the competitive ELISA and 0.031 to 20 ug/ml in the double-sandwich ELISA.

1. Indirect ELISA

a. Detection of Antibody Activity Towards NAGase

An indirect ELISA was performed for the detection of antibody activity of chicken IgY and rabbit IgG towards NAGase. A microtitre plate was coated with 20 µg/ml NAGase in 0.05 M sodium carbonate buffer, pH 9.6, and stored overnight at 4°C. Following incubation the coating solution was removed by shaking the plate contents into a sink. PBS Blotto (200 ul per well) was added and the plates incubated for 30 min. Blocking solution was removed and the antibody samples diluted with PBS Blotto were added to the wells. PBS Blotto containing no sample was added to 8 wells for blanking (control). Plates were incubated then washed with tap water. Anti-chicken - ALP conjugate diluted 1/2000 in PBS Blotto was added and the plates incubated. Following a final wash, enzyme substrate (0.1% p-nitrophenyl phosphate in diethanolamine buffer) was added and incubated for 30 min. The absorbance of each well at 405 nm was read using an SLT

Labinstrument (Austria) ELISA reader. Control values were subtracted from sample values.

b. Determination of the Working Dilution of Anti-NAGase - Alkaline Phosphatase Conjugates

An indirect ELISA, similar to the one described above, was used to determine the working dilutions of prepared anti-NAGase ALP conjugates. The following exceptions were made. Following the blocking and incubation steps, dilutions of anti-NAGase - ALP conjugates ranging from 10^{-1} to 10^{-5} in PBS Blotto were added. Control wells contained no conjugate. Following incubation the enzyme substrate was added.

2. Competitive ELISA

A competitive ELISA similar to that described by Huang et al. (1987) was performed, with modifications, for the detection of NAGase. A microtitre plate was coated with 20 µg/ml NAGase in 0.5 M sodium carbonate buffer, pH 9.6, and stored overnight at $4^{o}C$. Wells for the control were not coated. Coating solution was removed and 250 µl PBS Blotto added to each well. After incubation for 30 min, the solution was removed and to each well prepared chicken anti-NAGase (0.1 mg/ml in PBS Blotto) added. Immediately following, NAGase standards were added. Following incubation and washing, 200 µl of anti-chicken IgG - ALP conjugate diluted 1/2000 in PBS Blotto was added and incubation repeated. Plates were washed and the substrate added. After incubation for 30 min., absorbance of each well at 405 nm was read. Control values were subtracted from sample values.

3. Double-Sandwich ELISA

A double-sandwich ELISA for the detection of NAGase in fish samples was used. A microtitre plate was coated with 100 µg/ml prepared rabbit anti-NAGase IgG and the plates incubated. Following removal of the coating solution, 200 µl of PBS Blotto was added and the plates incubated. Blocking solution was removed and PJ and FE samples or NAGase standards added. Control wells contained no fish samples or NAGase. After incubation and washing, prepared chicken anti-NAGase (20 µg/ml in PBS Blotto) was added. Incubation and washing steps were repeated followed by addition of anti-chicken IgY ALP conjugate. Incubation and washing steps were repeated again and the enzyme substrate added. Twenty minutes later, absorbance at 405 nm was read. Control values were subtracted from sample values. Linear regression was performed on the data obtained from the standards. Concentration of NAGase in the fish samples was calculated using the standard curve.

F. Radial Immunodiffusion

Immunodiffusion plates were prepared using the method described by Milford-Ward (1981) with modifications. In one test-tube, 0.35 ml of rabbit anti-chicken IgG (Sigma, St. Louis, MO) was mixed with 1.65 ml of 0.2 M PBS, pH 7, and placed in a 55^{o}C water bath. In a second test-tube 0.07 g agarose (Sigma, St. Louis, MO) was mixed with 4.6 ml of PBS and 0.4 ml of a 0.35% NaN_3 solution and placed in a boiling water bath until the agarose dissolved. The contents of both

test-tubes were mixed and poured into radial immunodiffusion plates (ICN, Cleveland, OH). Following solidification, 3 mm diameter holes were cut in the agarose using a template (ICN, Cleveland, OH).

A standard curve was derived for each plate using chicken IgG (Sigma, St. Louis, MO) ranging in concentration from 0.2 to 1.2 mg/ml. Five microlitres of samples and standards were applied to the wells. Diffusion was allowed to proceed for 3 days in a closed Petri plate containing moistened filter paper. IgG concentrations were determined by comparing the square of the diameter of the precipitin ring (measured with a micrometre) versus the IgG concentration on the standard curve.

G. SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed using method B already described in PART I, with modifications. Samples were diluted in sample buffer to give a protein concentration of 2 mg/ml. Ten microlitre samples were applied to the gels. For analysis of chicken anti-NAGase IgY, chicken IgG (Sigma, St. Louis, MO) was used as a standard.

H. Statistical Analysis

A one-way analysis of variance was performed to determine if there was a significant difference in NAGase concentrations between fresh and frozen PJ samples.

A two-way analysis of variance was performed to determine if there was a significant difference in NAGase

concentration ratios between sample dilutions and between fresh and frozen salmon samples.

In the development of a standard curve for the double-sandwich ELISA for NAGase, linear regression was performed on the data. To determine if the line was significantly linear, the correlation coefficient (r) was calculated and compared to critical r values (Crow et al., 1978). The coefficient of determination (r^2) was also calculated. To determine the variation about the regression line, the standard error of estimate (SEE) was also calculated.

RESULTS AND DISCUSSION

PART I: PRODUCTION OF MONOCLONAL ANTIBODIES (Mabs) SPECIFIC FOR AN ENTEROPATHOGENIC E.COLI (EPEC)

A. Production of Specific Mab Secreting Hybridomas

1. Hybridoma Production

The first step in the production of monoclonal antibodies (Mabs) is the establishment of hybridomas which secrete the specific antibody desired. Hybridomas were produced using the fusion procedure described. Two fusions were performed on two separate occasions. Fox-NY myeloma cells were fused with spleen cells from mice immunized with the outer membrane of an enteropathogenic E.coli (EPEC). Fox-NY cells are deficient not only in HGPRT, but also adenosine phosphoribosyl transferase (APRT) (Taggart and Samloff, 1983). This allows the use of an alternate selection system using adenine, aminopterin and thymidine, thus allowing the survival of hybridomas that lose the X chromosome which codes for HGPRT during HAT selection (Eshhar, 1985). Under this selection system, hybridomas formed between a spleen cell and a myeloma cell that did not lose this X chromosome can utilize either exogenous adenine and thymidine or hypoxanthine and thymidine in a salvage pathway for nucleotide synthesis. Therefore, the selection media used in this experiment contained both aminopterin, hypoxanthine, adenine and thymidine.

After approximately 10 days of growth, the hybridoma clones were visible under a light microscope. Figure 4

depicts the hybridoma clones in a single well of a microwell plate. At this stage of growth the wells were tested for specific antibody production by ELISA. The E.coli OM preparation was used as the screening antigen. Table I shows the number and percentage of positive wells obtained in each fusion. An absorbance value of 0.1 was arbitrarily selected as the cut-off point for positive clones.

In fusion 1, of the 480 wells tested for antibody production, 11 (2.3%) tested positive. In fusion 2, of the same number of wells tested, 51 (10.6%) were positive for specific antibody production. Using this fusion protocol, albeit with a different antigen, as high as 80% of the wells screened have been reported to be positive (Wieczorek,1989). Lane et al. (1986) reported that while highly immunized spleens contain thousands of stimulated B-cells, less that 1% of these will fuse to become antibody-secreting hybridomas. This number has been shown to depend on the antigen dose and the immunogenicity of the antigen. Increases in immunizing antigen concentration tends to increase the yield of hybridoma clones producing specific antibody (Shibier et al., 1988). If the antigen is a poor immunogen, antibody response will be poor. The immunization protocol also seems to affect the yield. Stahli et al. (1983) showed that the specific efficiency, or yield of positive hybridomas increased up to 3 days following immunization, then decreased. Olsson et al. (1983) stated that B-cells should be in a certain stage of differentiation for successful

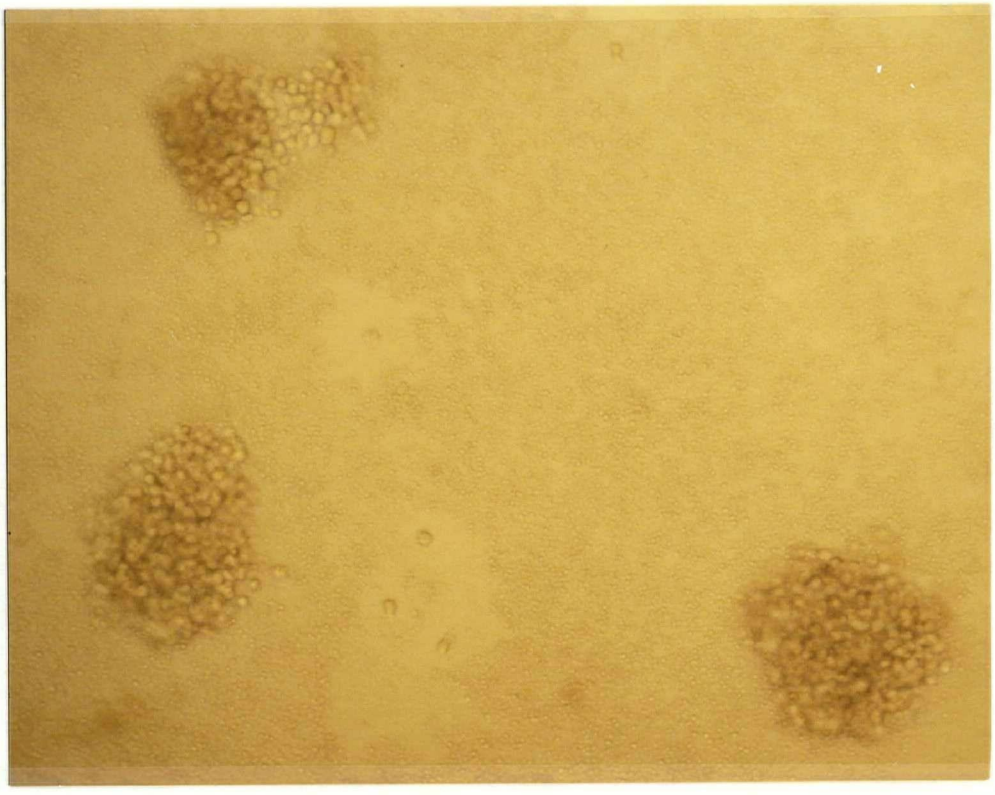

Figure 4: Hybridoma clones as seen under a light microscope (bright field) in a single well of a microwell plate after approximately 10 days of growth in AHAT media. (400X magnification)

Table I: Number and Percentage of Wells Containing Specific Antibody Producing Hybridomas

	Fusion	
	1	2
# of wells tested[a]	480	480
# of specific wells[b]	11	51
% of specific wells[c]	2.3%	10.6%

a. Five microwell plates were used to grow the culture from each fusion (96 wells/plate).

b. Those wells having an $A_{405} > 0.1$ when tested by ELISA

c. The percentage of wells containing clones specific for the antigen.

hybridization. There are also some reports that in vitro immunization is more efficient in terms of specific antibody production than immunization in vivo (Eshhar, 1985)

Ultimately the success of a fusion is determined by the number of hybridomas secreting Mab with the desired specificity and other properties. In this experiment the final selection of Mab secreting hybridomas was determined by cross-reactivity patterns with other bacteria.

Since it was practically impossible, due to limited time and manpower, to proceed further with all of the positive clones, a working number was selected based on those wells exhibiting the highest titres (eg. highest absorbance values) since this was a desirable property. From fusion 1, hybridomas from 4 wells were expanded and their reactivity confirmed by ELISA. These were then stored in liquid nitrogen. From fusion 2, hybridomas from 17 wells were expanded and their reactivity confirmed by ELISA. These were also stored in liquid nitrogen.

2. Recloning

After the identification and selection of positives, the next step was to reclone the hybridomas in the wells. Since the original positive wells often contained more than one clone of hybridoma cells (Figure 4), the desired cells could be outgrown by cells that were not producing the antibody of interest. Recloning ensures monoclonality and eliminates undesirable hybridomas. In this experiment recloning was performed by diluting the cells in a single positive well in

a microwell plate such that only 1 cell/well was present. Subsequent growth led to a single clone per well as determined by microscopic examination

Table II shows the results of recloning. All 4 cultures preserved from fusion 1 were recloned, while only 5 of the 17 from fusion 2 were recloned. On further expansion, only 4 of the 5 selected from fusion 2 grew in culture. A total of 49 single hybridoma clones were found to be specific for the E.coli OM by ELISA from fusion 1. From fusion 2, only 34 single clones were found to be specific for the antigen. Again, since it was impossible to test all of the positive clones, only 8 were selected from each fusion based on high titres in ELISA. Since the absorbance readings for positive clones were generally higher than the readings obtained prior to recloning, a higher value (A_{405}) was chosen as the cut-off point for selection. The total of 16 hybridomas selected consisted of two from each original positive well from each fusion. These hybridoma cultures were recorded and stored in liquid nitrogen.

3. Immunofluorescence Screening

Specificity for the antigen (OM) was not the only property for which the Mab were selected. In this experiment, patterns of cross-reactivity with other bacteria was an important selection criterion. To test for cross-reactivity, Mabs from the recloned hybridomas were screened against a panel of Enterobacteriaceae including both EPEC and non-EPEC strains. All of the bacterial strains were obtained

Table II: Numbers of Positive Wells Selected for Recloning and Numbers of Resulting Positive Single Clones

	Fusion	
	1	2
# of selected positive wells[a]	4	17
# selected for recloning	4	5
# of single positive clones[b] after recloning	49	34

a. Those positives exhibiting high titres prior to recloning

b. Wells containing a single clone by microscopic examination and having an $A_{405} > 0.5$ in ELISA

from the British Columbia Provincial Health Laboratory and were isolated from stool specimens of both adults and infants. EPEC 018:K77 did not grow when transfered to a TSA slant and therefore was excluded from the panel. Testing was done by indirect immunofluorescence assay using undiluted supernatant fluid from hybridoma cultures as the antibody source. Whole cells were used as the antigens. Six hybridoma cultures from the 16 selected reclones were randomly chosen for screening. The results are qualitative since neither the antibody nor antigen concentration were standardized.

Table III shows the results of the immunofluorescence screening. For this experiment a Mab which was specific to all EPEC yet did not react with other Enterobacteriaciae, including non-EPEC, was desirable. The data shows that the most promising antibody in this respect was 2F9 B3. It was positive for all the EPEC strains, showed minimal cross-reactivity with other Enterobacteriaceae, and except for #3, did not cross-react with the non-EPEC strains on the panel. However, under the conditions used in this assay the fluorescence observed was relatively weak, including that for the positive control. Some cells appeared to stain and some did not, while others were only partially stained. Positive reactions with Mabs 3A5 C10, 2E1 H6 and 5E10 B6 had a similar fluorescent image. This may be due to low concentrations of antibody present in the supernatant fluid of the hybridomas. It is also possible that these Mabs are

Table III: Results of the Preliminary Screening of Selected Mabs Against a Panel of Enterobacteriaceae by Immunofluorescence Assay using Supernatant Fluid From Hybridoma Cultures.

	Mabs						
Bacteria	3A5C10	2H4H12	4D10C1	2E1H6	5E10B6	2F9B3	C[a]
EPEC O142:K86:H6[b]	+[c]	+++	+++	+	+	+	-
EPEC O128:K67	+	-	-	+	+	+	+
EPEC O55:K59	+	-	+	+	+	+	-
EPEC O44:K74	+	-	-	+	+	+	+
EPEC O112:K68	+	+	-	+	-	+	-
E.coli (#1)[d]	-	-	-	+	-	-	-
E.coli O157:H7	+	-	-	+	-	-	-
E.coli O157:K88:H19	-	-	-	+	-	-	-
E.coli (#2)	+	-	-	+	-	-	-
E.coli (#3)	+	-	-	+	+	+	-
S.marcescens	+	+	-	-	-	+	+
C.freundii	+	-	-	+	+	-	-
E.cloacae	+	-	+	-	+	-	-
E.tarda	-	-	-	-	-	-	-
K.pneumoniae	+	-	-	+	+	-	-
P.mirabilis	+	-	-	-	-	-	-
P.morganii	-	-	+	-	+	-	-
H.alvei	+	-	-	-	+	-	-
E.fergosonii	-	-	-	-	-	-	-
E.hermanii	+	-	-	-	+	-	-
P.rettgeri	+	-	-	+	+	-	-
K.ascorbita	+	-	-	-	+	-	-
Alkalescens-dispar-I	+	-	-	-	+	-	-

a. negative control
b. positive control
c. fluorescence intensity is on a scale of - to +++
d. no serotype information was available for numbered E.coli

specific for only a minor antigen on the surface of the bacteria or its antigenic determinant is not fully exposed. The weak fluorescence observed in these reactions may make detection of these bacteria difficult.

Mab 2E1 H6 appears to be specific for a determinant common to E.coli since it reacted with all of the E.coli on the panel, yet reacted with only two non-E.coli strains. This antibody may therefore have potential in detecting E.coli as a group.

Both Mabs 3A5 C10 and 5E10 B6 exhibited extensive cross-reactivity with the bacteria tested and therefore, were omitted from further studies. Mabs 2H4 H12 and 4D10 C1 were selected for further study since both were restrictive in their cross-reactivities. In addition, the fluorescence observed when they reacted with the positive control was strong. This indicates that these antibodies are almost solely specific for the E.coli strain to which they were raised and that the antigenic determinant to which the antibodies bind was not common to most of the bacteria on this panel.

The results obtained from screening the antibodies against EPEC 0128:K67, EPEC 044:K74 and S. marcescens were inconclusive since the negative controls exhibited some fluorescence. This may be due to nonspecific capture or attachment of antibodies to the bacteria surface.

B. Batch Production and Purification of Mabs

Large amounts of the selected antibodies were obtained by growing the hybridomas in vivo as ascitic tumours in mice. Eight pristane-treated mice were injected intraperitoneally with the selected hybridoma cultures (2 mice for each culture). After 7 days, the abdomens of the mice had swollen sufficiently to be "tapped" for ascites fluid. Ascites production yielded 30 ml of fluid containing Mab 2H4 H12, 14 ml of 2E1 H6, 7 ml of 4D10 C1 and 9 ml of 2F9 B3 before the mice expired.

Mabs usually only represent 10% of the total protein content of the ascites fluid (Bruck et al., 1986). Other proteins present include transferrin, albumin and proteases (Kohler and Milstein, 1975) as well as endogenous immunoglobulins (Burchiel, 1986). Therefore attempts were made to purify the Mab in the ascites fluid. Both gel filtration and anion-exchange chromatography were tried.

Mouse ascites fluid containing Mab 2E1 H6 showed a single major protein peak when separated on a Sephacryl S-300 SF column (Figure 5). A small shoulder appeared at the leading edge of this peak suggesting the presence of another unresolved peak. Purification of ascites fluid containing Mab 2F9 B3 showed a similar profile (Figure 6), although a peak near the void volume of the column (fraction 14 or 28 ml) was more apparant in this case. The elution profile of ascites fluid containing Mab 4D10 C1 shows a relatively clear separation of two peaks (Figure 7). Separation of ascites

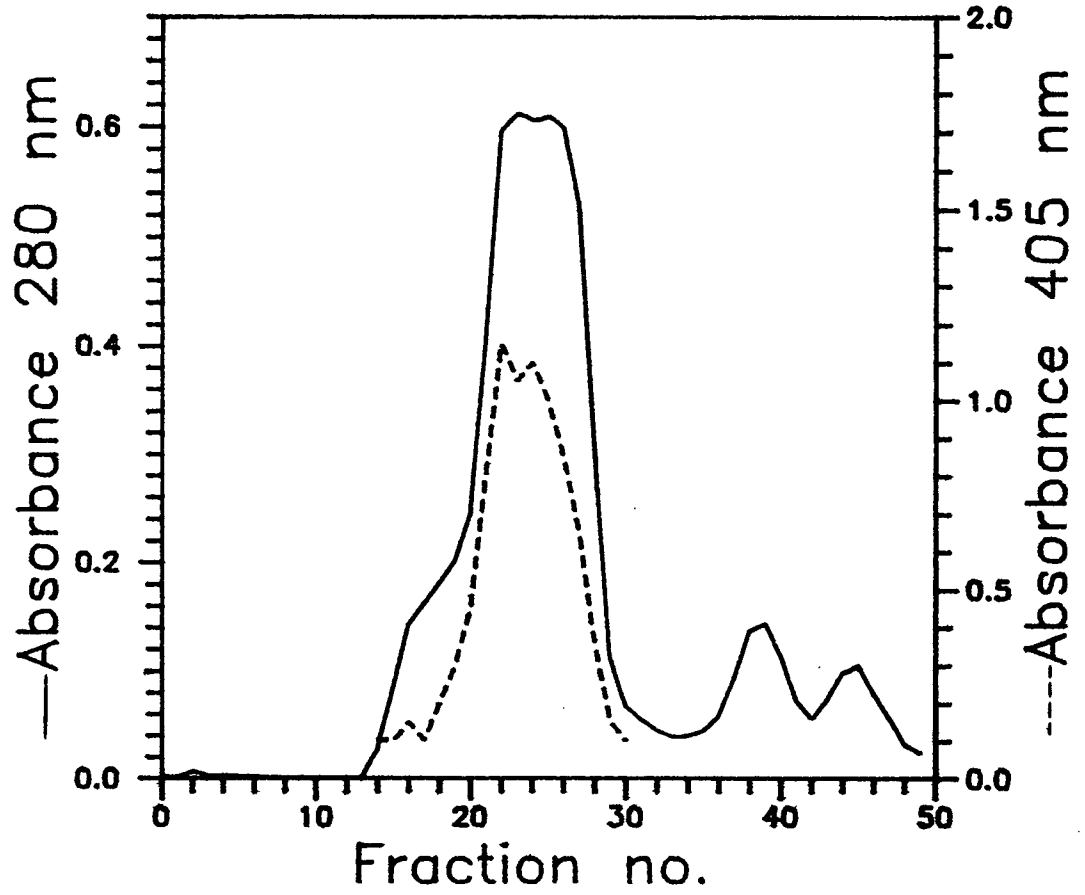

Figure 5: Elution profile of Mab 2E1 H6 separated by gel filtration chromatography. Immunoreactivity as determined by an indirect ELISA is also shown. Column: Sephacryl S-300 (1.8 X 34 cm); Eluting buffer: 0.1 M Tris-HCl, pH 8, with 0.5 M NaCl; Flow rate: 2 ml h^{-1}; Fractions: 2 ml.

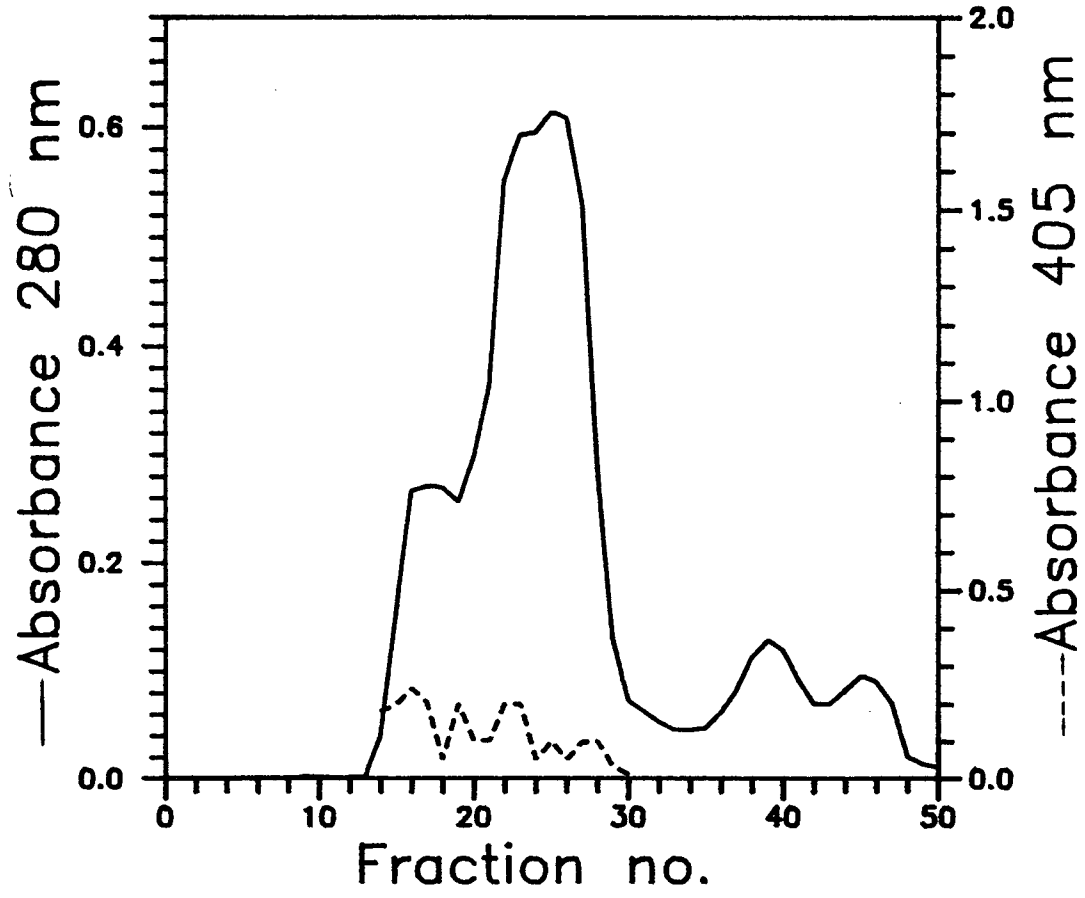

Figure 6: Elution profile of Mab 2F9 B3 separated by gel filtration chromatography. Immunoreactivity as determined by an indirect ELISA is also shown. Column: Sephacryl S-300 (1.8 X 34 cm); Eluting buffer: 0.1 M Tris-HCl, pH 8, with 0.5 M NaCl; Flow rate: 2 ml h^{-1}; Fractions: 2 ml.

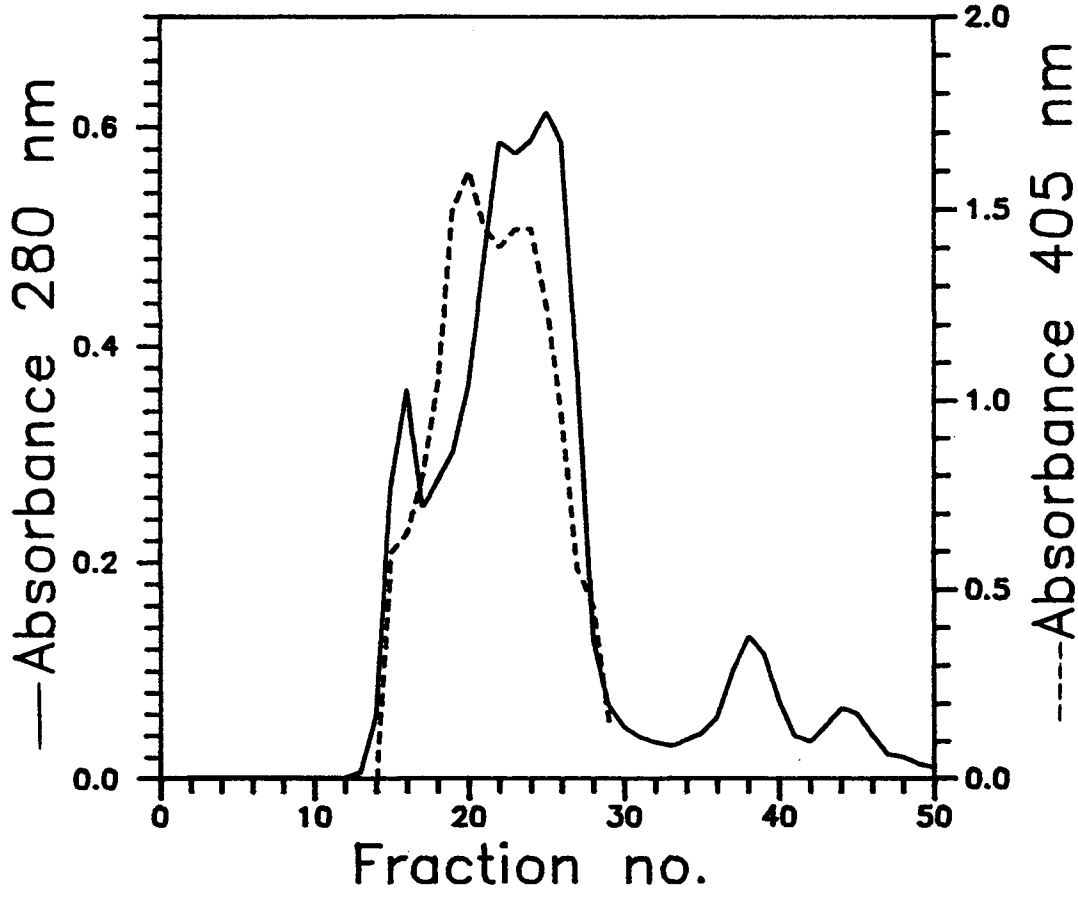

Figure 7: Elution profile of Mab 4D10 C1 separated by gel filtration chromatography. Immunoreactivity as determined by an indirect ELISA is also shown. Column: Sephacryl S-300 (1.8 X 34 cm); Eluting buffer: 0.1 M Tris-HCl, pH 8, with 0.5 M NaCl; Flow rate: 2 ml h^{-1}; Fractions: 2 ml.

fluid containing Mab 2H4 H12 shows the same two peaks, with the first peak being much larger than in the other purified ascites fluids (Figure 8). In the elution profiles of 2E1 H6, 2F9 B3 and 4D10 C1 the main protein peak appears to be doubly peaked, suggesting that two unresolved peaks were present. In the elution profile of 2H4 H12 this could also be seen in the presence of a small shoulder in the second peak eluted.

The presence of the antibody peak was determined by reacting the various fractions with the E.coli OM preparation in an ELISA. Figures 5 and 7, show strong immunoreactivity in the main peak, indicating the presence of the Mab in this peak. Results from Figure 6 are inconclusive since immunoreactivity was poor in all fractions. This is possibly due to the presence of low concentrations of Mab or the Mab present had a poor affinity for the antigen. Assay of fractions from purified Mab 2H4 H12 showed strong immunoreactivity in the first eluted peak (Figure 8). Immunoreactivity was also observed in the second peak. This suggests the presence of two antibody classes with significantly different molecular weights. Since the first antibody eluted near the void volume, it was presumed to be of the IgM class which has an approximate molecular weight of 900 kilodaltons (kD) (Clezardin et al., 1986).

Eluted peak fractions were subjected to electrophoresis under reducing conditions on a 10% SDS-polyacrylamide gel and the separated proteins identified by Coomassi staining. Figures 9 and 10 show the SDS-PAGE profiles of selected column

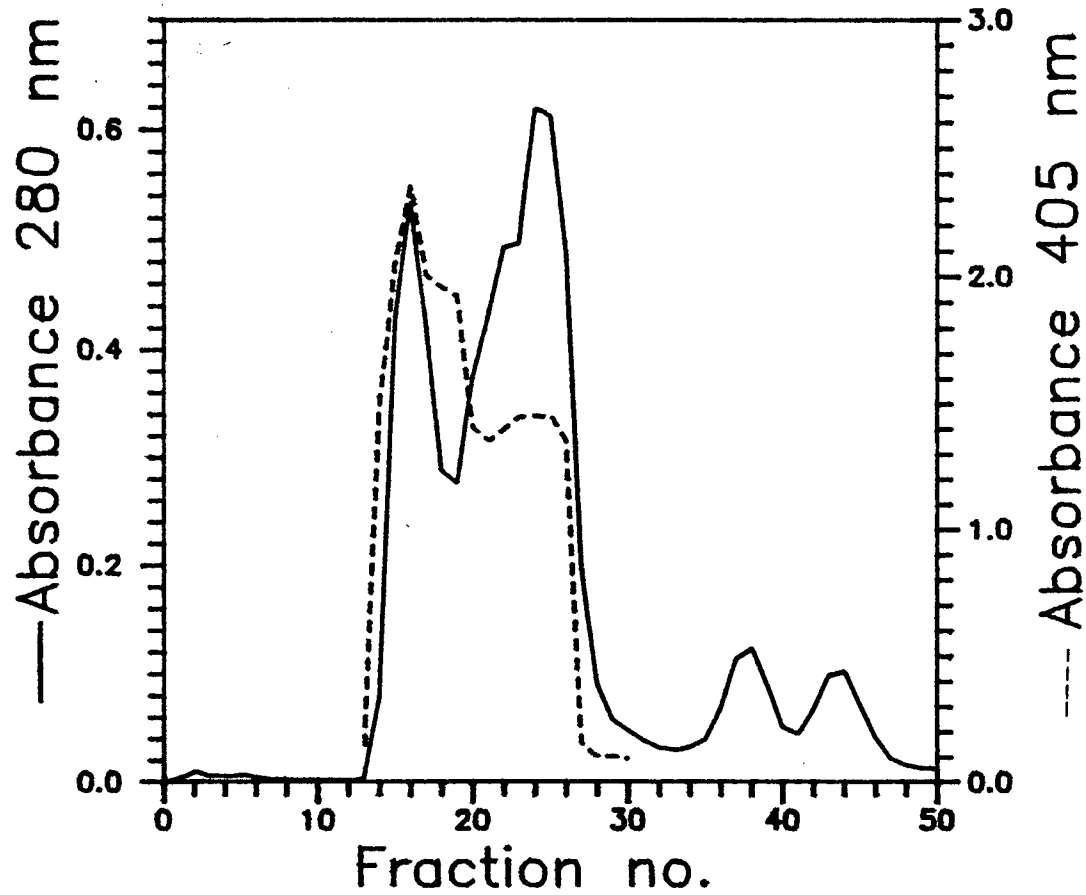

Figure 8: Elution profile of Mab 2H4 H12 separated by gel filtration chromatography. Immunoreactivity as determined by an indirect ELISA is also shown. Column: Sephacryl S-300 (1.8 X 34 cm); Eluting buffer: 0.1 M Tris-HCl, pH 8, with 0.5 M NaCl; Flow rate: 2 ml h^{-1}; Fractions: 2 ml.

fractions. In Figure 9, lanes 2-5 show the profiles of fractions from the purified ascites fluid containing Mab 2E1 H6. No major protein bands were observed in fractions 16 and 20 which correspond with the shoulder observed near the void volume. Fraction 22 showed 3 major protein bands at approximately 60, 50 and 25 kD. The 50 and 25 kD bands correspond to the apparent molecular weights of the heavy chain and light chain of the IgG antibody (Brodeur and Tsang, 1986). The 60 kD band was presumably albumin which represents the largest fraction of ascitic proteins (Franek, 1986) and typically migrates to this position in SDS-PAGE (Clezardin, 1986). Fraction 24 contained significantly more albumin in addition to some IgG. Similar profiles were observed for purified fractions of ascites fluid containing Mab 2F9 B3 (Figure 9). The fact that albumin co-eluted with the antibodies confirms the presence of two unresolved peaks.

The first peaks, which eluted near the void volume of the column, may be 2 macroglobulin which is a common contaminant of ascites fluid and has a molecular weight of approximately 900 kD (Fahey and Terry, 1969). Because of its high molecular weight it would not be resolved on the polyacrylamide gel used in this study.

Figure 10 shows the electrophoretic profiles of fractions from the purified ascites fluid containing Mab 2H4 H12. Fraction 16 showed 3 major protein bands. Due to the early elution of the protein peak, the antibody present was

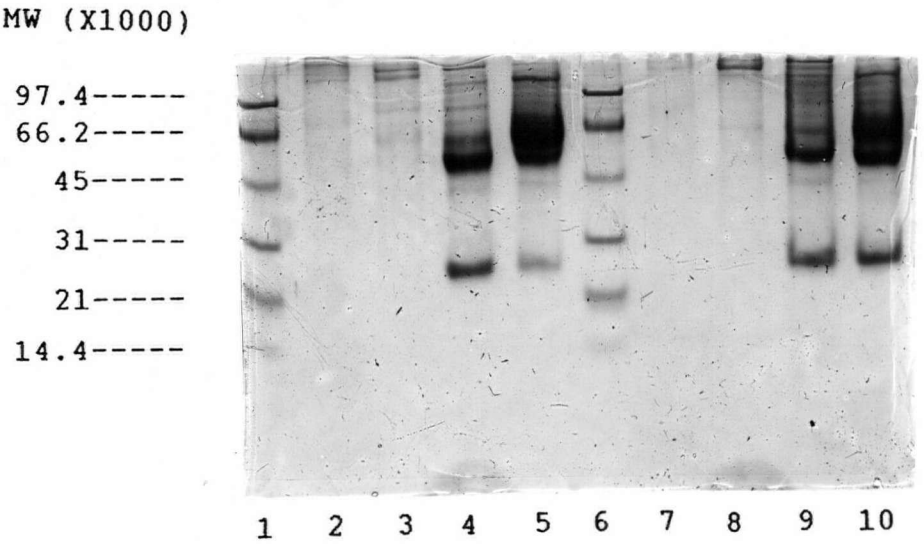

Figure 9: SDS-PAGE profiles of 2-ME-reduced samples from gel filtration purification. Lanes 1 and 6: molecular weight standards; Lanes 2-5: 2E1 H6 fractions 16, 20, 22, 24; Lanes 7-10: 2F9 B3 fractions 16, 20, 22, 24.

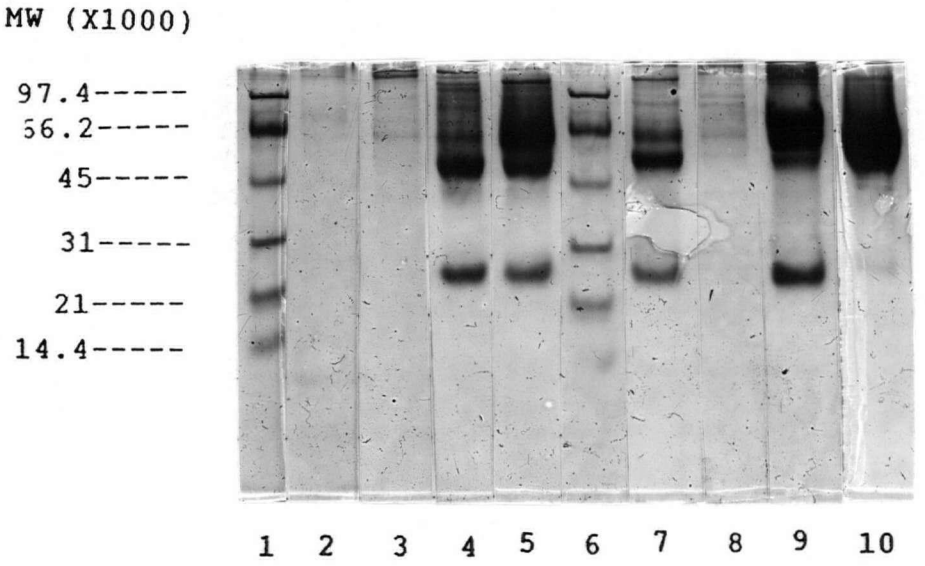

Figure 10: SDS-PAGE profiles of 2-ME-reduced samples from gel filtration purification. Lanes 1 and 6: molecular weight standards; Lanes 2-5: 4D10 C1 fractions 16, 20, 22, 24; Lanes 7-10: 2H4 H12 fractions 16, 20, 22,24.

presumed to be of the IgM class. The first band was expected to be IgM heavy chain since it has been shown to have a higher molecular weight and therefore, a slower mobility than IgG heavy chain (Ghebrehiwet, 1986). The second band, with a molecular weight similar to IgG heavy chain may indicate the presence of this Ig class. Some IgG molecules present may have interacted with the IgM molecules so as to cause them to co-elute. If this were the case, the third protein band would consist of both IgM and IgG light chains. Although it was not shown in this gel, the antibody may have been contaminated with α2-macroglobulin which has been shown to elute with IgM in gel filtration because of their similar size (Fahey and Terry, 1969). Fraction 22, which corresponded to the shoulder observed in the second peak, showed the presence of albumin in addition to IgG heavy and light chain. Fraction 24, which corresponded to the second peak, appeared to contain primarily albumin.

Given that each hybridoma produces an antibody of a single class (Burnet, 1957) it would appear that two different hybridomas were injected into the mice during ascites production of Mab. In other words, recloning was not successful in isolating a single clone in this case. Either the number of clones was miscalculated or at the time of counting the clones the second clone was not visible due to slow growth. However, under conditions of ascites production, it was given full advantage to grow. This may account for the presence of two different antibody classes in

the ascites fluid. It is also possible that the second antibody was endogenous. Gooi and Feizi (1982) found that in some instances, normal serum from untreated mice showed substantially higher binding to determinants on the antigen (fetal glycoproteins) than did the ascites fluid sample containing Mab specific for the antigen. Generally, however, the amount of polyclonal mouse Ig is very low in ascites fluid (Franek, 1986). In this study, SDS-PAGE results indicated that the concentrations of the two antibodies were similar (Figure 10).

Another explanation for the presence of the two classes may be that of class switching. A small percentage of switch variant hybridomas have been shown to spontaneously switch from producing one class or subclass of antibody to another while in culture. For example, Spira et al. (1984) reportedly raised a hybridoma which produced IgM that bound to phosphorylcholine, but also found IgG in the culture. This IgG was also able to bind the antigen.

Regardless of the explanation for the presence of the two classes, fractions 14-18, corresponding to the first peak (Figure 8) were pooled, concentrated to 1 ml and termed Mab 2H4 H12. This antibody fraction was selected since it was the purer of the two and showed a higher immunoreactivity with the antigen.

While IgG has been successfully purified by gel filtration (Wieczorek, 1989), resolution was poor under the conditions of this study. Rather than attempting to modify

the conditions of gel filtration, the use of anion-exchange for the purification of Mabs was investigated using ascites fluids containing Mabs 2F9 B3, 2E1 H6 and 4D10 C1.

Mouse ascites fluid containing Mab 4D10 C1 showed 3 major protein peaks when separated on a DEAE-Sephacel column with a gradient of 0 - 1 M NaCl (Figure 11). A fourth peak appeared at fraction 7 indicating a protein contaminant which did not bind to the column. This peak was present in all 3 samples purified. Analysis of the fractions eluted under the gradient by ELISA showed the antibody fraction to be located in the first peak. This peak was eluted at a salt concentration of approximately 0.5 M NaCl. The second and third peaks eluted at a salt concentration of 0.65 M and 0.8 M NaCl respectively.

Figure 12 shows the elution profile of ascites fluid containing Mab 2F9 B3. Again, 3 major protein peaks appeared under the elution conditions. However, the first two peaks were unresolved. The first peak appeared to elute at approximately 0.55 M NaCl while the second peak eluted at 0.65 M NaCl. The third peak was much larger in this sample and eluted at 0.8 M NaCl. An examination of the fractions by ELISA indicated a low immunoreactivity, although it was predominantly in the first of the two unresolved peaks.

In the purification of ascites fluid containing Mab 2E1 H6 only 2 major peaks were eluted (Figure 13). The first peak eluted at 0.65 M NaCl, consistent with the second peak observed in the two previous ascites samples. The second

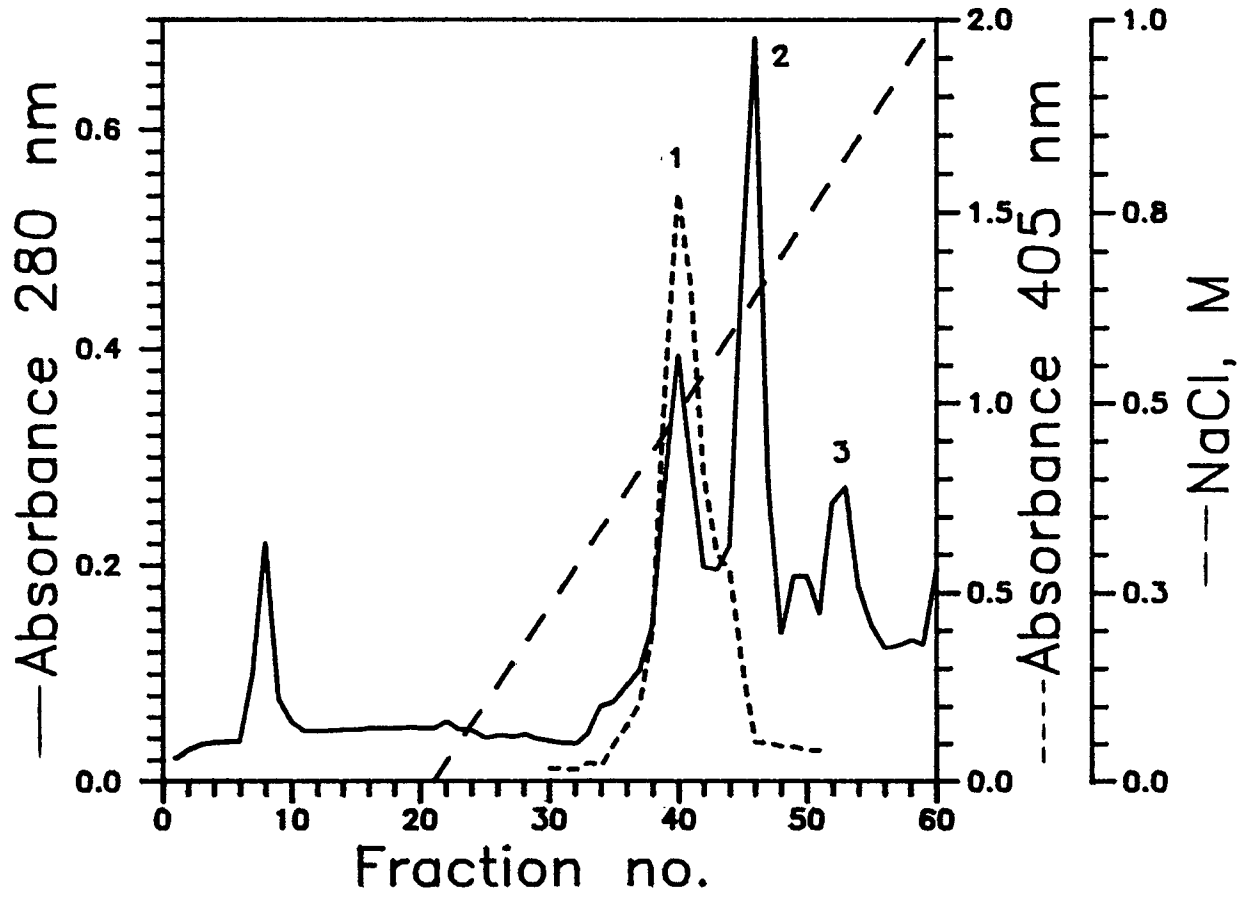

Figure 11: Elution profile of Mab 4D10 C1 separated by anion-exchange chromatography. Immunoreactivity as determined by an indirect ELISA is also shown. Column: DEAE-Sephacel (1.8 X 7 cm); Eluting buffer: 10 mM Tris-HCl, pH 8, NaCl concentration gradient; Flow rate: 5 ml h^{-1} ; Fractions: 1 ml.

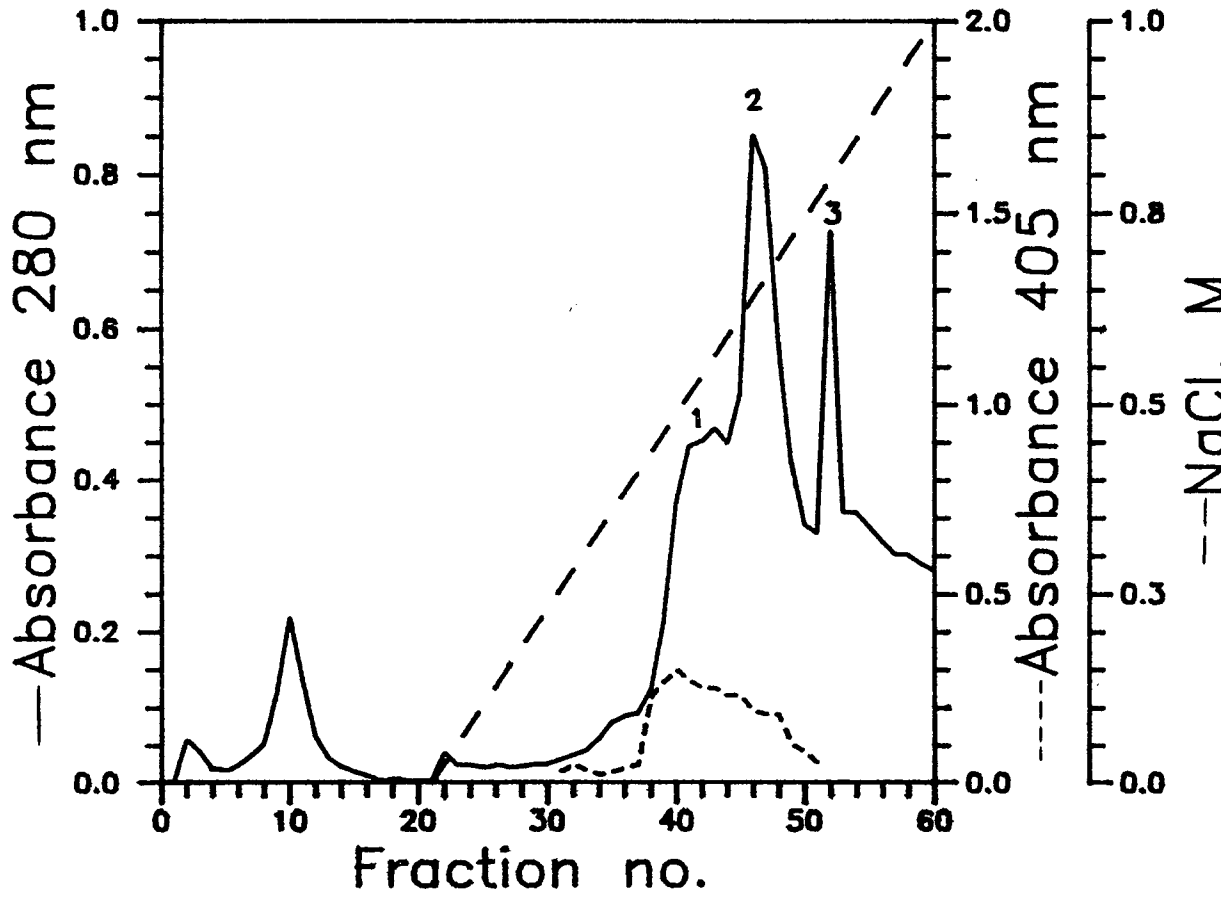

Figure 12: Elution profile of Mab 2F9 B3 separated by anion-exchange chromatography. Immuno-reactivity as determined by an indirect ELISA is also shown. Column: DEAE-Sephacel (1.8 X 7 cm); Eluting buffer: 10 mM Tris-HCl, pH 8, NaCl concentration gradient; Flow rate: 5 ml h^{-1}; Fractions: 1 ml.

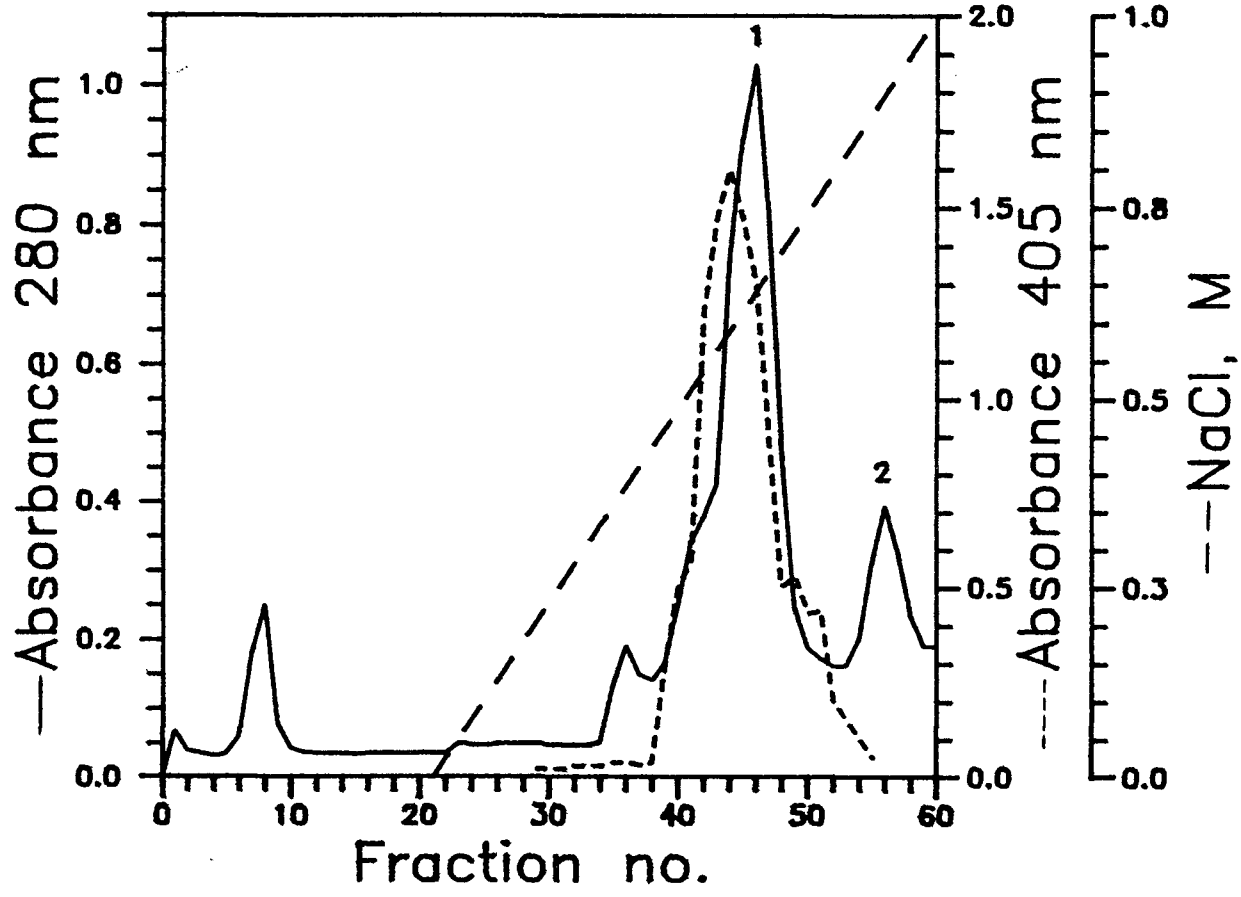

Figure 13: Elution profile of Mab 2E1 H6 separated by anion-exchange chromatography. Immunoreactivity as determined by an indirect ELISA is also shown. Column: DEAE-Sephacel (1.8 X 7 cm); Eluting buffer: 10 mM Tris-HCl, pH 8, NaCl concentration gradient; Flow rate: 5 ml h^{-1}; Fractions: 1 ml.

peak eluted close to 0.9 M NaCl. ELISA showed the antibody fraction to be located in the second peak.

The peak fractions were subjected to electrophoresis under reducing conditions. Figures 14 and 15 show the SDS-PAGE profiles of selected fractions of purified mouse ascites fluids. In Figure 14, lanes 2-4 show the profiles of fractions from the purified ascites fluid containing Mab 2F9 B3. The antibody was located in fraction 40 which corresponded to the first peak in the elution profile (Figure 12), and was evident by the heavy and light chains of IgG at approximately 50 and 25 kD respectively. Another protein band appeared at 66 kD. This corresponded to the apparent molecular weight of transferrin which commonly co-elutes with IgG in anion-exchange chromatography (Burchiel et al., 1984). Fraction 45, which corresponded to the second peak, showed a large protein band between 50 and 65 kD. This was presumably comprised predominantly of albumin. No bands appeared in fraction 51, probably due to a low protein concentration or the inability of the gel to resolve the protein. Lanes 6-8 show the profiles of fractions from the purified ascites fluid containing Mab 2E1 H6. Fraction 41, which corresponded to the shoulder observed at the leading edge of the main peak (Figure 13), showed a single protein band which was probably transferrin. Fraction 46, which corresponded to the main peak, showed bands corresponding to the heavy and light chains of Ig in addition to albumin. Fraction 56, from the third peak, contained no bands.

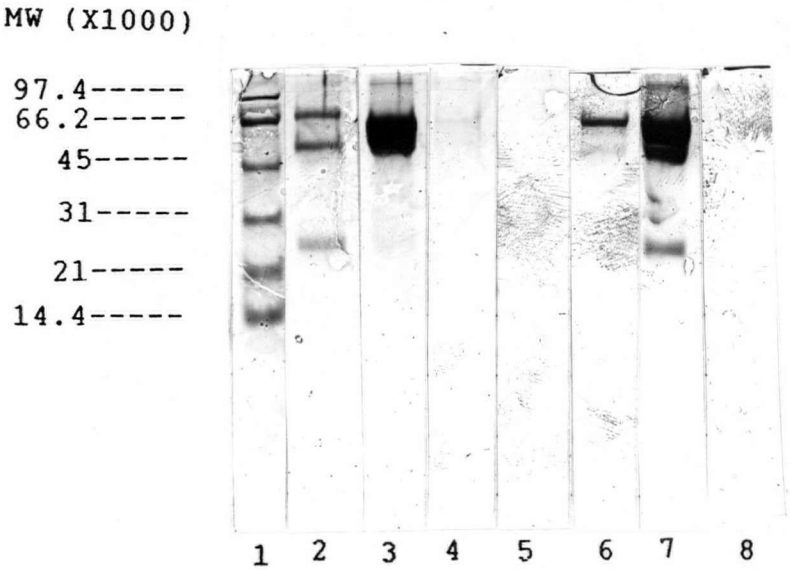

Figure 14: SDS-PAGE profiles of 2-ME-reduced samples from ion-exchange purification. Lane 1: molecular weight standards; Lanes 2-4: 2F9 B3 fractions 40, 45, 51; Lanes 6-8: 2E1 H6 fractions 41, 46, 56.

Figure 15 shows the profiles of fractions from the purified ascites fluid containing Mab 4D10 C1. Fraction 40, corresponding to the first peak, clearly showed the presence of the heavy and light chains of Ig in addition to transferrin. The second peak consisted primarily of albumin which was shown by the heavy protein band in fraction 46. Fractions 50 and 53 showed no major protein bands.

Mabs 4D10 C1 and 2F9 B3 appeared to have a similar charge density since they eluted at similar salt concentrations. On the other hand, Mab 2E1 H6 appeared to have a similar charge density to albumin which explains their co-elution. In fact, Burchiel et al. (1984) already demonstrated that different monoclonal mouse IgGs elute at different times under identical conditions. In addition, Horejsi and Hilgert (1986) mentioned that IgG Mabs can differ markedly in their isoelectric points and so would have different charge profiles at similar pHs. The elution conditions for the optimal separation of monoclonal IgG molecules from ascites fluid are different for each antibody. In this situation, it may be advantageous to use purification procedures that separate on the basis of properties such as size, not change greatly between different antibodies. Optimization of gel filtration conditions for separation of the Mabs may prove to be a better procedure in this respect.

The transferrin that co-eluted with the Mab in anion-exchange is often removed in a second purification step, usually gel filtration (Burchiel et al., 1984). This was not

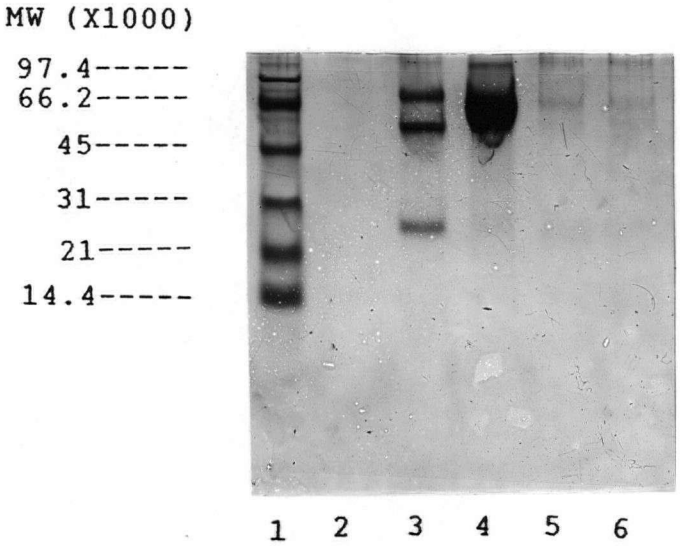

Figure 15: SDS-PAGE profiles of 2-ME-reduced samples from ion-exchange purification. Lane 1: molecular weight standards; Lanes 3-6: 4D10 C1 fractions 40, 46, 50, 53.

done in this experiment since purity was not a critical factor. For the same reason, further purification of Mab 2E1 H6 was not attempted despite its' co-elution with albumin.

Fractions from the peaks containing immunoglobulin were pooled and concentrated to 1 ml.

C. Characterization of the Mabs and their Antigens

The purified Mabs were characterized on the basis of their immunoreactivity with the antigen, isotype and specificity. Information on their respective antigens was obtained by their reaction in an immunoblot assay.

1. Immunoreactivity

The immunoreactivity or antigen-binding capacity of the Mabs was determined using an indirect ELISA. OM preparation of E.coli was used as the antigen. Since the specific concentration of immunoglobulin was not known, reactivity was determined on a total protein basis. Figure 16 shows the results. From this graph the titres (the minimum protein concentration at which significant protein reactivity was observed) can be compared. Neither Mab 2E1 H6 nor 2F9 B3 showed very high reactivity even at the highest protein concentration tested (0.01%). Mabs 4D10 C1 and 2H4 H12 exhibited a markedly higher reactivity even at concentrations as low as 0.0001% in the case of 4D10 C1. Concentrations above 0.001% were not tested for Mabs 4D10 C1 and 2H4 H12 due to the low protein concentrations of these Mab preparations as well as their limited supply.

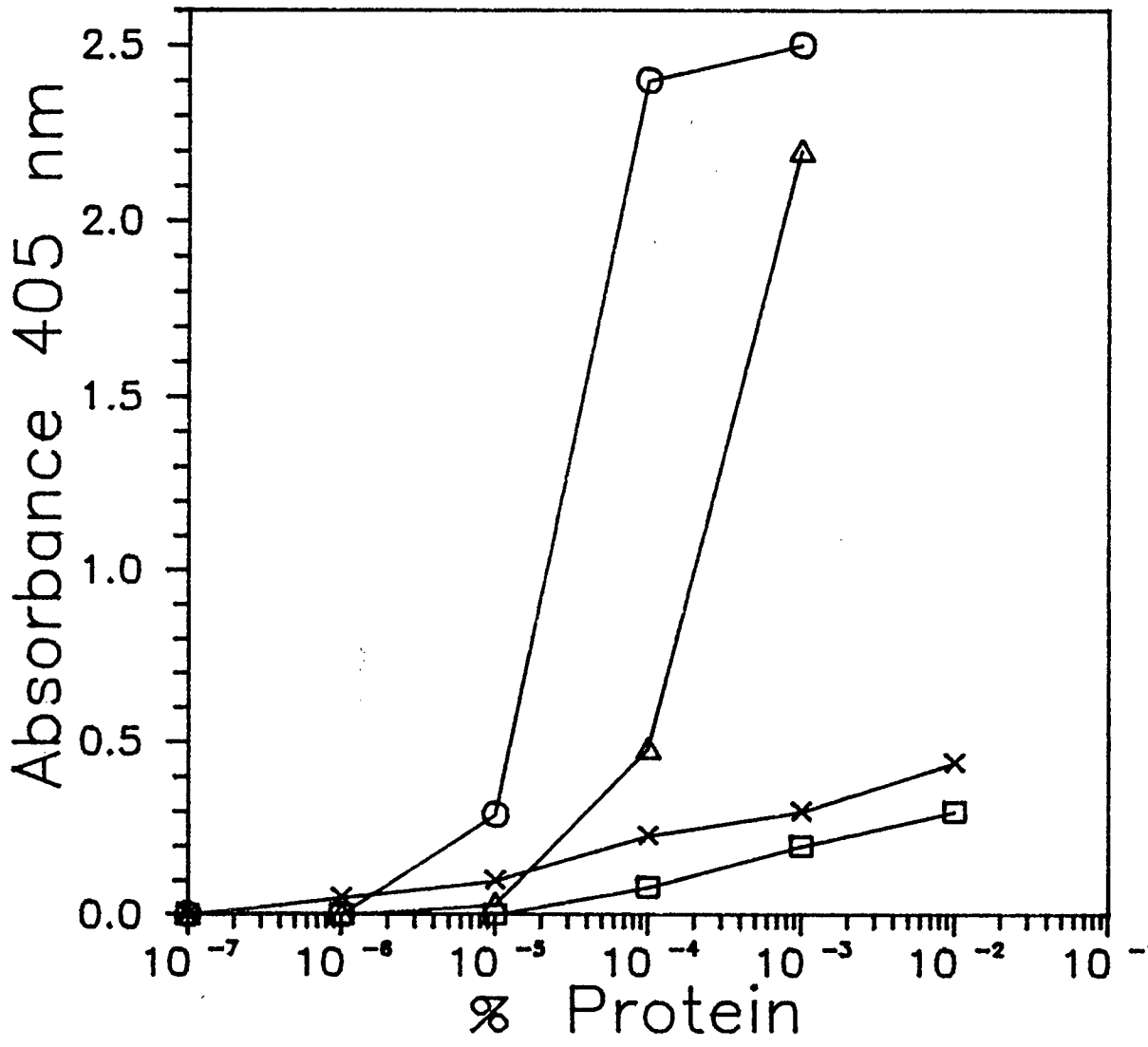

Figure 16: Antigen binding activity of Mabs as determined by an indirect ELISA. Mab 2E1 H6 (□); 2F9 B3 (X); 4D10 C1 (△); 2H4 H12 (○).

The low reactivity observed for Mabs 2E1 H6 and 2F9 B3 may be due to a low concentration or a low affinity of these Mabs for the antigen. Another explanation may be that the antigenic determinants they are specific for represent only a small portion of the OM. This could result in less antibody binding and therefore, a lower signal than if more determinants were present.

2. Isotype Analysis

To determine the class or subclass of the Mabs, they were reacted with anti-mouse class specific antibodies in an indirect ELISA. The results are shown in Table IV. Both Mab 2E1 H6 and Mab 2F9 B3 reacted positively with the anti-IgG1 antibody. Mab 4D10 C1 reacted positively with both anti-IgG2a and anti-IgG2b, suggesting that both subclasses were present in the purified antibody preparation. This would imply that an error was made in recloning and monoclonality was not achieved. However, as was mentioned previously, the possibility exists that the hybridoma producing these antibodies was a subclass switch variant. Preud'homme et al. (1975) observed that IgG2b producing myeloma cells often spontaneously switch to expressing IgG2a molecules. This may account for the presence of both antibody subclasses.

Mab 2H4 H23 reacted positively with anti-IgM and anti-IgG3. This confirms the presence of these two classes which was already elucidated from the OD_{280} profile of this Mab when purified by gel filtration (Figure 8).

Table IV: Results of Isotyping of Mabs

Mab	Ig Isotype[a]
2F9 B3	IgG1
2E1 H6	IgG1
4D10 C1	IgG2a, IgG2b
2H4 H12	IgM, IgG3

a. Anti-isotype specific antibodies with which the Mabs reacted positively in an indirect ELISA.

3. Immunofluorescence Screening

Mabs were screened against a panel of bacteria to test their specificity. An indirect immunofluorescence assay, as was done for the hybridoma culture fluids previously, was used. Table V shows the results of the screening.

Both 2H4 H12 and 4D10 C1 showed very little cross reaction with the bacteria on the panel. Mab 2H4 H12 cross-reacted weakly with EPEC O112:K68 and K.pneumoniae. Mab 4D10 C1 cross reacted weakly with EPEC O55:K59, E.cloacae and P.morganii. This would suggest that these bacteria possess antigenic determinants which are close enough in structure to the true determinant so as to bind weakly to the antibody. The weak fluorescence may also be due to non-specific staining. Non-specific staining is a result of non-immunologic attachment of antibodies to the bacteria or to the presence of natural antibodies in the Mab preparation which cross-react with determinants on the bacteria (Bigazzi and Tilton, 1980). Also, since optimum dilutions of Mab were not determined, it is possible that too high concentrations were present. High concentrations of antibody may lead to an increase in cross-reactions (Borrebaeck and Glad, 1989).

Cross-reaction of Mabs 2E1 H6 and 2F9 B3 with the bacteria was extensive. Both reacted with all the E.coli serotypes in addition to many of the Enterobacteriaceae on the panel. This would suggest the presence of a common or similar antigenic determinant in the outer membrane of these bacteria. The pattern of cross-reactivity was much different

Table V: Results of the Second Screening of Selected Mabs Against a Panel of Enterobacteriaceae by Immunofluorescence assay using Purified Mabs.

Bacteria	Mabs				
	2H4 H12	4D10 C1	2E1 H6	2F9 B3	C[a]
EPEC O142:K86:H6[b]	+++[c]	+++	++	++	-
EPEC O128:K67	-	-	++	++	-
EPEC O55:K59	-	+	++	++	-
EPEC O44:K74	-	-	++	++	-
EPEC O112:K68	+	-	++	++	-
E.coli (#1)[d]	-	-	++	++	-
E.coli O157:H7	-	-	++	++	-
E.coli O157:K88:H19	-	-	++	++	-
E.coli (#2)	-	-	++	++	+
E.coli (#3)	-	-	++	++	-
S.marcescens	-	-	++	++	-
C.freundii	-	-	++	++	-
E.cloacae	-	+	++	++	-
E.tarda	-	-	-	-	-
K.pneumoniae	+	-	++	++	-
P.mirabilis	-	-	+	-	-
P.morganii	-	+	++	++	-
H.alvei	-	-	-	++	-
E.furgosonii	-	-	-	-	-
E.hermanii	-	-	+	-	-
P.rettgeri	-	-	-	-	-
K.ascorbi	-	-	-	-	-
Alkalescens-dispar-I	-	-	+	-	-

a. negative control
b. positive control
c. fluorescence intensity is on a scale of - to +++
d. no serotype information was available for numbered E.coli

than that observed when hybridoma culture fluid was used as the antibody source (Table III). The concentration of Mab may have been too low in the hybridoma culture fluid to stain some cells. This would also explain the weaker fluorescence seen in those positive samples as compared to the same samples in the second screening. It is also possible that the specificity of the Mab changed during growth in ascites. Cross-reactivities in some Mabs have been shown to differ markedly when raised in ascites as compared to the same Mab raised in culture (Bosch et al., 1982). Mutations have been shown to occur in the immunoglobulin gene of some cultured antibody-producing cells (Cotton et al., 1973; Bruggeman et al., 1982). If the growth environment of the cells is altered significantly, such as in ascites growth, the mutant may develop a growth advantage and outgrow the original cell population. Immunoglobulins produced by the mutant could show different specificities (Bosch et al., 1982). Another explanation may be that monoclonality was not achieved during recloning and a second hybridoma producing antibody with a different specificity was present during growth in ascites. Due to slow growth this hybridoma may not have been producing sufficient antibody in hybridoma culture to significantly influence immunofluoresence results. Growth of this hybridoma may have been stimulated in ascites and therefore, more of this antibody was produced. This may have contributed to the change in the immunofluorescence results.

4. Immunoblot Analysis

Immunoblotting was carried out to gain information on the antigen itself. Three EPEC, 3 non-EPEC and 6 non-E.coli strains were randomly selected from the bacteria screening panel for analysis. SDS-PAGE profiles of cell lysates were obtained then transfered electrophoretically to a polyvinylidene difluoride membrane. Replicates were prepared and each allowed to react with a different Mab in an ELISA. Figure 17 depicts the electrophoretic profiles of the cell lysates under reducing conditions. Figures 18 to 21 depict the immunoblots of the bacteria samples with Mabs 2E1 H6 and 2F9 B3. Both were strongly reactive with a protein band in the control EPEC with an apparant molecular weight of approximately 35 kD (Figure 18). This band was common to all the EPEC tested. A second band appeared at approximately 30 kD in the 2E1 H6 and 2F9 B3 blots of the control EPEC, but not in the other EPEC blots.

Figure 19 shows the immunoblots of non-EPEC with the same Mabs. Again, the Mabs reacted with a 35 kD protein band. A 30 kD band was also visible in the non-EPEC blots.

In Figure 20 it can be seen that C.freundii, E.cloacae, and K.pneumoniae contained a cross-reactive protein of approximately the same molecular weight as the 35 kD protein in the control in both Mab blots. In addition a 30 kD cross-reactive protein was also present. In the 2F9 B3 blot of the control two bands appeared, one at 30 kD and another at 28 kD. Since the 28 kD band did not appear in previous blots of

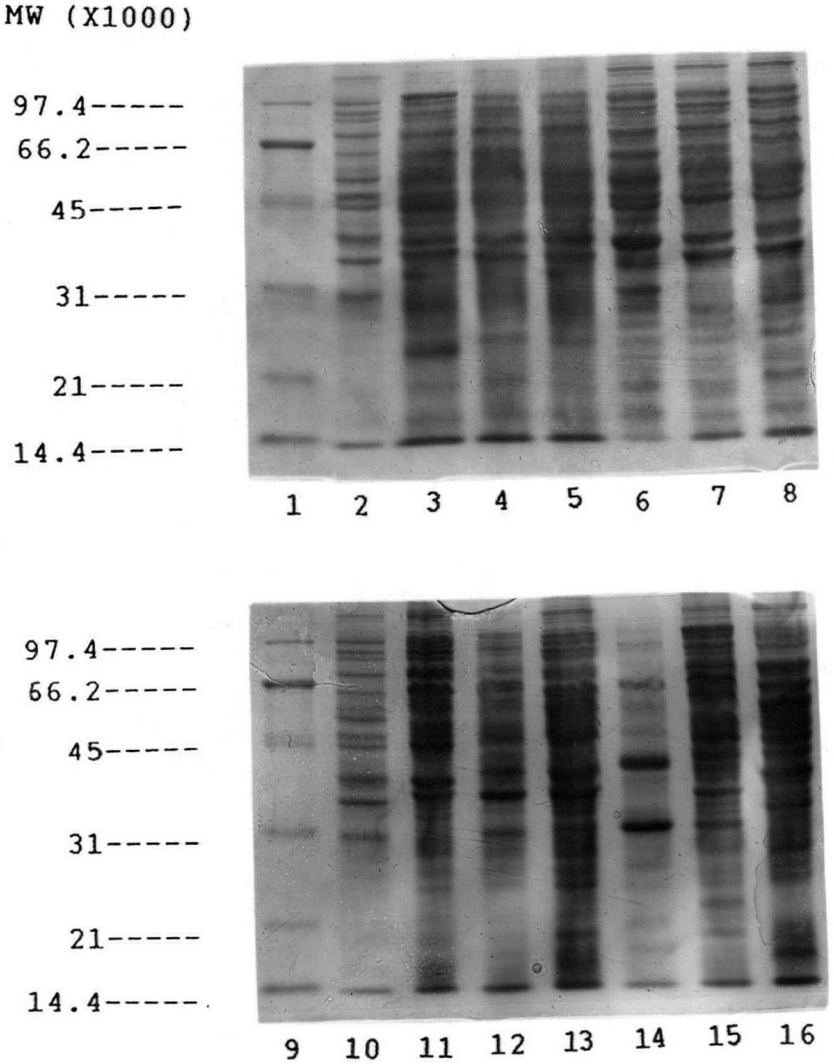

Figure 17: SDS-PAGE profile of 2-ME-reduced bacteria samples. Lanes 1 and 9: molecular weight standards; Lanes 2 and 10: EPEC O142:K86:H6; Lane 3: EPEC O128:K67; Lane 4: EPEC O55:K59; Lane 5: EPEC O44:K74; Lane 6: E.coli O157: H7; Lane 7: E.coli O157:K88:H19; Lane 8: E.coli, non-EPEC; Lane 11: C.freundii; Lane 12: E.cloacae; Lane 13: K.pneumoniae; Lane 14: S.marcescens; Lane 15: E.hermanii; Lane 16: P.mirabilis.

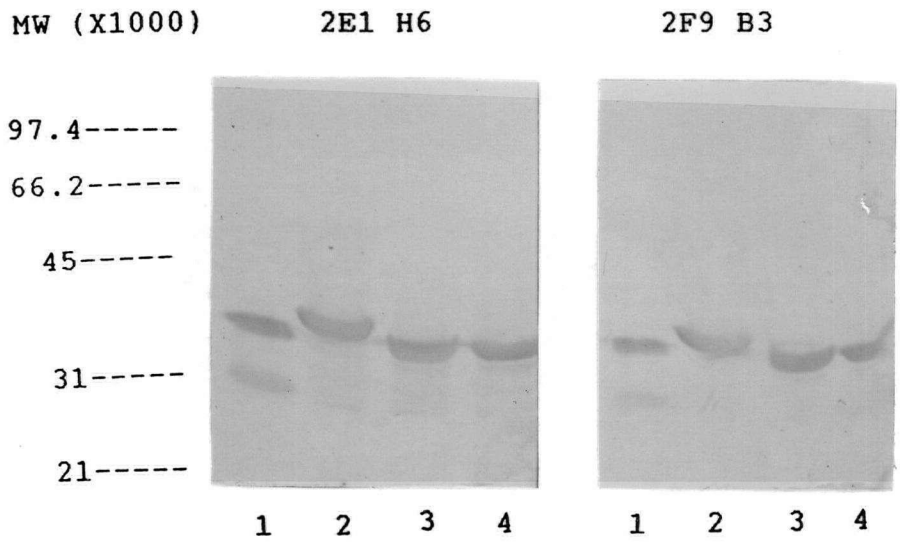

Figure 18: Immunoblots of EPEC with Mabs 2E1 H6 and 2F9 B3. Lane 1: EPEC O142:K86:H6 (control); Lane 2: EPEC O128:K67; Lane 3: EPEC O55:K59; Lane 4: EPEC O44: K74.

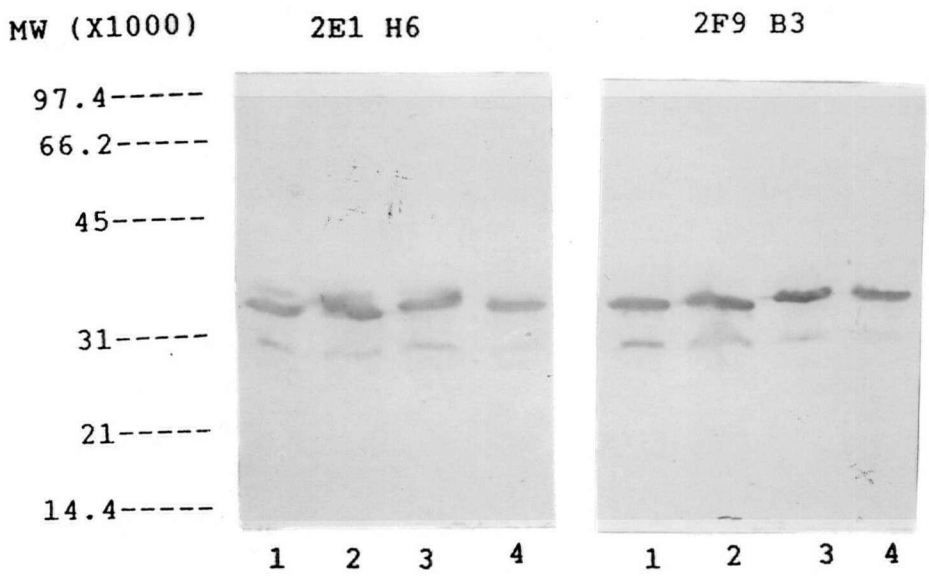

Figure 19: Immunoblots of non-EPEC with Mabs 2E1 H6 and 2F9 B3. Lane 1: EPEC O142:K86:H6 (control); Lane 2: E.coli O157:H7; Lane 3: E.coli O157:K88: H19; Lane 4: E.coli, non-EPEC.

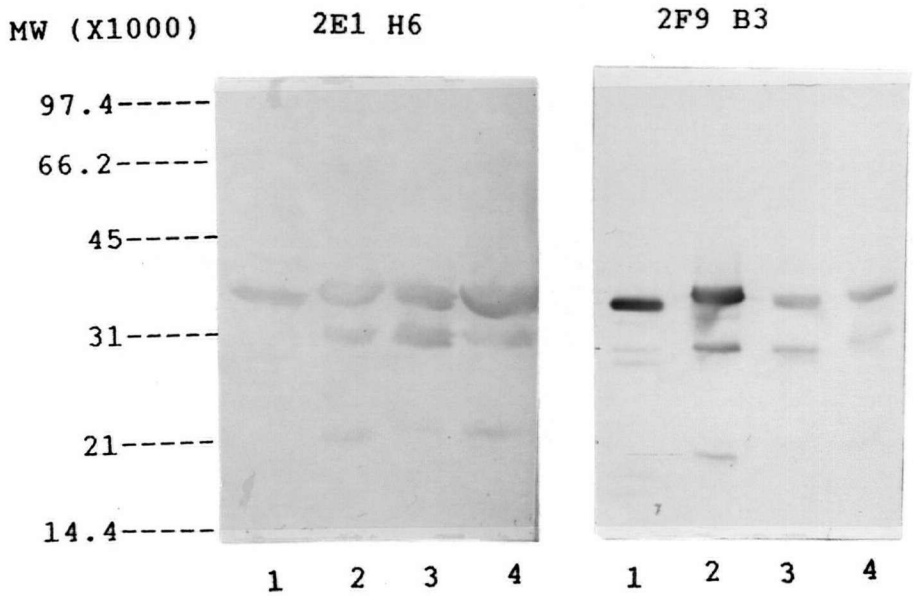

Figure 20: Immunoblots of Enterobacteriaceae with Mabs 2E1 H6 and 2F9 B3. Lane 1: EPEC O142:K86:H6 (control); Lane 2: C.freundii; Lane 3: E. cloacae; Lane 4: K.pneumoniae.

the control it was not considered to be a major cross-reaction. The 30 kD band did not appear in the 2E1 blot of the control or the control blots of Figure 21. This may be due to inefficient transfer of this protein during blotting. Alternatively, it may reflect the instability of this protein under SDS-denaturing conditions. A faint band of approximately 20 kD also appeared in the 2F9 B3 blot of C. freundii and the 2E1 H6 blot of C.freundii and K.pneumoniae. This probably represents a very minor cross-reaction. In the 2E1 H6 blot of S.marcescens (Figure 21), only a 30 kD cross-reactive protein band appeared. E.hermanni contained both a 35 kD and a 30 kD protein which were cross-reactive with 2E1 H6. Mab 2E1 H6 was not reactive with P.mirabilis. Mab 2E1 H6 did not cross react with S.marcescens, E.hermanii or P.mirabilis, suggesting that these bacteria do not share common or similar determinants with the control E.coli.

It cannot be stated whether the cross-reacting proteins observed in the bacteria were the same as those found in the control EPEC. In spite of their similar electrophoretic mobilities, they may be different proteins with shared or similar antigenic determinants.

The extensive cross-reactivity pattern observed with Mabs 2E1 H6 and 2F9 B3 among the bacteria panel was not unusual since antigenic cross-reactivity among the outer membrane proteins is a common phenomenon in the Enterobacteriaceae family (Hofstra and Dankert, 1980). The

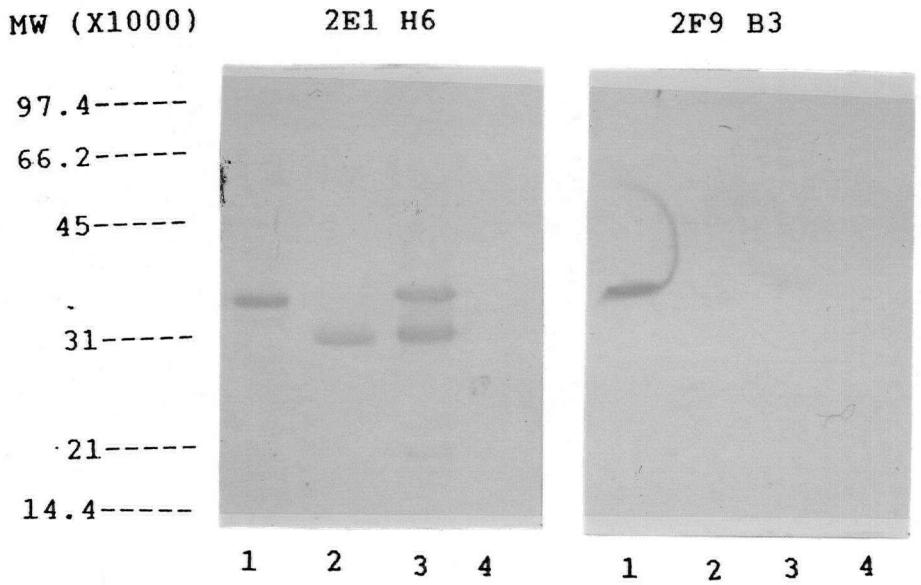

Figure 21: Immunoblots of Enterobacteriaceae with Mabs 2E1 H6 and 2F9 B3. Lane 1: EPEC O142:K86:H6; Lane 2: S.marcescens; Lane 3: E.hermanii; Lane 4: P.mirabilis.

cross-reactive 35 kD protein observed in nearly all the blots corresponded closely to the electrophoretic mobility of the heat-modified form of the outer membrane protein (Omp) A which has been well characterized in E.coli K12 (Lambert, 1988). Proteins cross-reactive with Omp A have previously been detected in all strains of E.coli in addition to many other Enterobacteriaceae (Hofstra and Dankert, 1980). It is possible that protein bands that appeared at 28 to 30 kD were unmodified portions of the same protein present due to insufficient heating. Hofstra and Dankert (1980) reported that the unmodified form of the proteins cross-reactive with Omp A protein in the family Enterobacteriaceae were in the molecular weight range of 26.5 to 31 kD.

Table VI compares the results of the immunoblots with those of the immunofluorescence assay. Aggreement between results was observed in all cases except with S.marcescens and P.mirabilis. Mab 2F9 B3 reacted positively with S.marcescens in the immunofluorescence assay, but reacted negatively in the immunoblot. Similarly, Mab 2E1 H6 reacted positively with P.mirabilis in the immunofluorescence assay, but negatively in the immunoblot. While these antibodies were capable of detecting the cross-reacting determinants in the whole cell, the determinants were seemingly not recognizable when denatured as on blots.

Neither Mab 4D10 C1 nor Mab 2H4 H12 reacted in the immunoblots (i.e. there were no visible reacting protein bands) either under reducing or non-reducing conditions.

Table VI: Results of Immunoblotting vs. Immunofluorescence Assay

Bacteria	Immunoblot		Immunofluoresence	
	2F9 B3	2E1 H6	2F9 B3	2E1 H6
EPEC 0142:K86:H6[a]	+[b]	+	+	+
EPEC 0128:K67	+	+	+	+
EPEC 055:K59	+	+	+	+
EPEC 044:K74	+	+	+	+
NON-EPEC 0157:H7	+	+	+	+
NON-EPEC 0157:K88:H19	+	+	+	+
NON-EPEC, not 0157	+	+	+	+
C.freundii	+	+	+	+
E.cloacae	+	+	+	+
K.pneumoniae	+	+	+	+
S.marcescens	-	+	+	+
E.hermanii	-	+	-	+
P.mirabilis	-	-	-	+

a. positive control
b. + = positive reaction
   - = no reaction

This is thought to be due to their inability to detect the antigenic determinant under SDS-denaturing conditions, since not all antigenic sites retain their native configuration after SDS treatment (Towbin and Gorden, 1984).

The increased specificity of Mabs over polyclonal antibodies can readily be seen by comparing immunoblots prepared with both monoclonals and polyclonals. Figure 22 depicts blots of various bacteria cell lysates reacted with polyclonal antiserum obtained from the egg yolks of chickens immunized with E.coli O142:K86:H6 (preparation of specific antibodies from egg yolk is discussed in Part II of this thesis). The multiple banding represents the presence of antibodies with varying specificities, which is typical of polyclonal antisera. By comparison, the Mabs exhibited a narrow range of specificity as shown by the limited number of reacting protein bands (Figures 18 to 21). Although the polyclonal antiserum was not tested for cross-reactivity with all of the Enterobacteriaceae on the panel, it is reasonable to expect the degree of cross-reactivity with these bacteria to be high since the presence of cross-reacting antigens among the family Enterobacteriaceae is also high (Hofstra and Dankert, 1980).

D. Conclusion

Under the conditions of this experiment there was no success in obtaining a Mab uniquely specific to EPEC. This does not imply that such a Mab does not exist among the antibody repertoire of the many hybridomas raised, only that

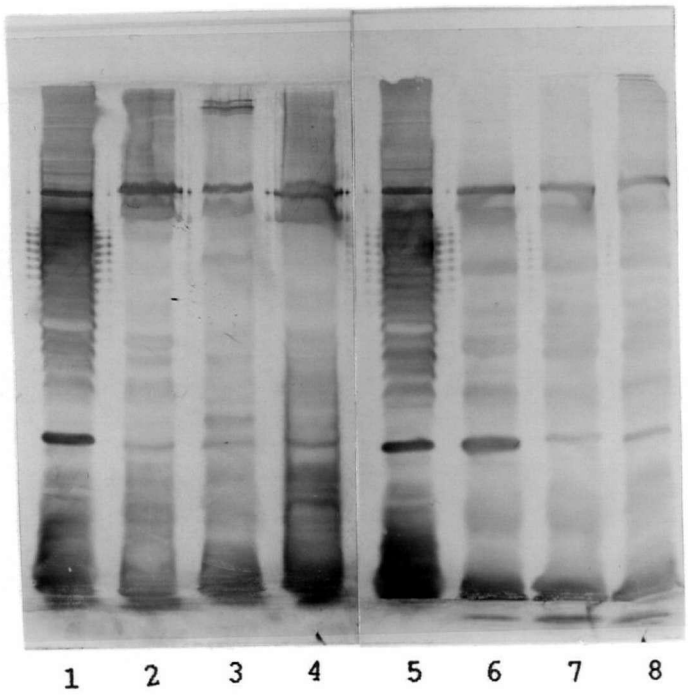

Figure 22: Immunoblot of EPEC and other Enterobacteriaceae with polyclonal antiserum. Lanes 1 and 5: EPEC O142:K86:H6 (control); Lane 2: EPEC O128:K67; Lane 3: EPEC O55:K59; Lane 4: EPEC O44:K74; Lane 6: C.freundii; Lane 7: E.cloacae; Lane 8: K.pneumoniae.

to the extent of screening performed in this study such an antibody was not found. Given sufficient time and manpower it is feasible that, if there is a uniquely specific surface antigen common only to EPEC, a complimentary Mab could be found. It is possible that the 94 kD OM protein expressed in some Class I EPEC strains, reported in the literature review, is such an antigen. Isolation of this protein and its use as the immunogen may vastly decrease screening time. A Mab specific for this protein may have potential use in an assay to distinguish this E.coli type from others.

Nevertheless, in this study two Mabs (4D10 C1 and 2H4 H12) were produced which were strongly reactive with a single EPEC serotype in an immunofluorescence assay, while showing minimal cross-reactivity with other bacteria. This Mab may have potential use in the detection of this E.coli serotype in the feces of infants or in food systems. Because of the strong fluorescence observed, this EPEC could easily be detected. A second pair of antibodies were also produced which were weakly reactive with all the E.coli serotypes tested in addition to most of the other Enterobacteriaceae on the panel. Immunoblotting showed these Mabs to be reactive in most cases with both a 35 kD and a 30 kD protein. It is possible that the heavier protein is similar to the 35 kD Omp A protein described in the literature (Lambert, 1988). These Mabs may have potential use in detecting bacteria belonging to the Enterobacteriaceae family.

Obviously further experiments testing for antigenic cross-reactivity among a larger panel of bacteria are required. In addition non-denaturing immunoblots with Mabs 4D10 C1 and 2H4 H12 should be performed in order to gain information on the reactive antigen. While Mabs 4D10 C1 and 2H4 H12 showed a strong affinity and 2E1 H6 and 2F9 B3 a weak affinity for the OM preparation in ELISA, it remains to be shown if they are reactive with the whole cell in this type of assay.

In using Mabs, one must be aware of the potential problems associated with them. Very often their stability to chemical and physical treatments, such as changes in pH and temperature, are less than that observed with polyclonal antibodies (Kurstak, 1986). As was shown in this thesis and has been reported previously (Bosch et al., 1982), the cross-reactivity profiles of the Mabs may change due to changes in the growth conditions of the hybridomas. In addition, some hybridoma clones that produce desired antibodies can spontaneously become non-producers (Young, 1984). Unless the antigen contains multiple identical determinants, precipitation reactions between Mabs and antigens generally do not occur (Galfre and Milstein, 1981). Because the Mab is specific for a single antigenic determinant, negative results do not necessarily imply absence of antigen since changes in the environment of the determinant or the way the antigen is presented could significantly alter results.

Despite these problems, however, monoclonal antibodies have significant advantages over polyclonal antibodies and will remain a powerful tool for many scientific disciplines, including food science.

PART II: USE OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO DETECT ß-N-ACETYLGLUCOSAMINIDASE (NAGase)

A. Production of Antibodies Specific for NAGase

Both White Leghorn hens and New Zealand white rabbits were injected with a commercial bovine ß-N-acetylglucosaminidase (NAGase) preparation. Immunoglobulin Y (IgY) was separated from the egg yolk of immunized chickens by polyethylene glycol precipitation followed by DEAE-Sephacel chromatography. This experiment was performed in triplicate. The Ig fraction of the blood serum from immunized rabbits was isolated by ammonium sulphate precipitation.

Purity of the isolated IgY was examined by SDS-PAGE and radial immunodiffusion. Figure 23 shows the SDS-PAGE profiles of the three fractions compared to standard IgY. All three fractions exhibited protein bands typical of the heavy and light chains of IgY. Radial immunodiffusion showed the three fractions of IgY to have a purity of between 80 and 82%.

Ammonium sulphate precipitation was the only purification step performed on the rabbit blood serum since it is reported that this method is sufficient to yield an IgG fraction of approximately 95% purity (Garvey et al. 1977). This method was performed on blood serum samples obtained from each of the two rabbits used in this experiment. Figure 24 shows the reduced SDS-PAGE profiles of the two fractions compared to molecular weight standards. A large protein band

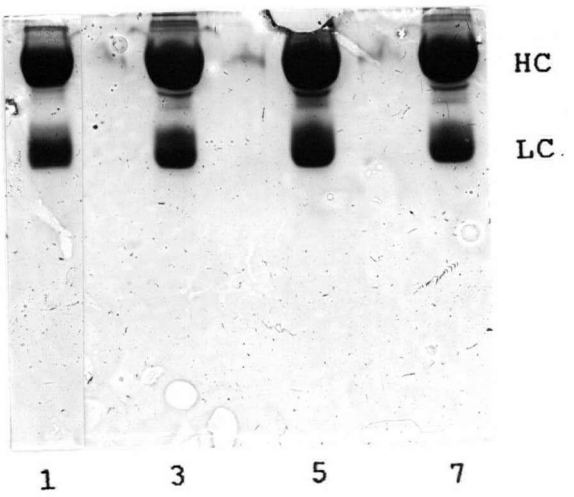

Figure 23: SDS-PAGE profiles of 2-ME-reduced purified IgY fractions. Lane 1: IgY standard; Lanes 3, 5 and 7: IgY fractions 1, 2 and 3. Heavy chains (HC) and light chains (LC) of Ig are shown.

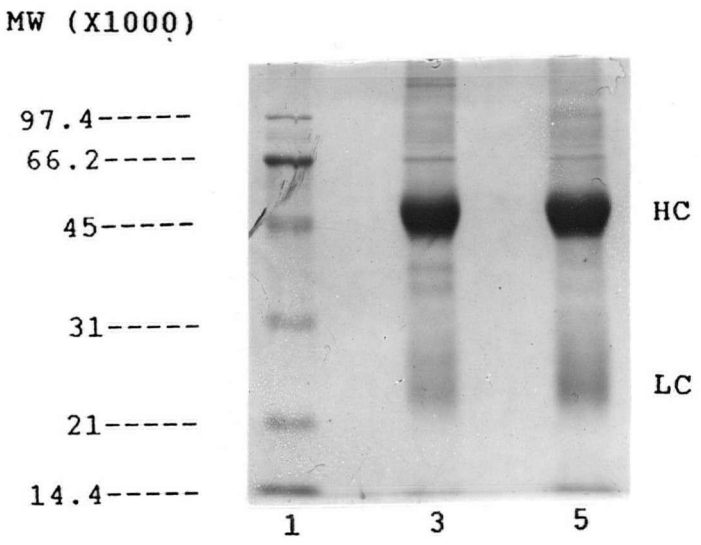

Figure 24: SDS-PAGE profiles of 2-ME-reduced purified rabbit IgG fractions. Lane 1: molecular weight standards; Lanes 3 and 5: IgG fractions from rabbits 1 and 2. Heavy chains (HC) and light chains (LC) of Ig are shown.

appears at approximately 45 kD. This value is close to the molecular weight of the heavy chain of rabbit IgG (Marler et al., 1964). A diffuse band also appeared at approximately 25 kD which corresponded to the molecular weight of the light chain of rabbit IgG (Marler et al., 1964). Other minor bands reflected the presence of some contaminating proteins. Radial immunodiffusion was not performed on the rabbit IgG fractions since anti-rabbit antibody and standard rabbit IgG were not available.

It has already been established by McCannel (1988) that chickens immunized with NAGase are capable of generating an antibody response to this immunogen. To verify this, the immunoreactivity of the IgY fractions against NAGase was tested. This was done using an indirect ELISA. IgY fractions at various protein concentrations were tested. Figure 25 shows the results. As the concentration of IgY was increased there was a subsequent increase in absorbance at 405 nm indicating an increase in reaction with NAGase. Above a concentration of 0.01% protein, the activity decreased. This is unusual since one would expect the absorbance values (and thus immunoreactivity) to reach a plateau once the binding sites on the NAGase bound to the microwell plate were saturated. Instead, higher concentrations seemed to promote a decrease in the antigen-antibody interaction. This phenomenon is not uncommon to ELISAs (de Savigny and Voller, 1980) and is considered to be a type of "prozone" effect. Prozone effects are generally observed in precipitation and

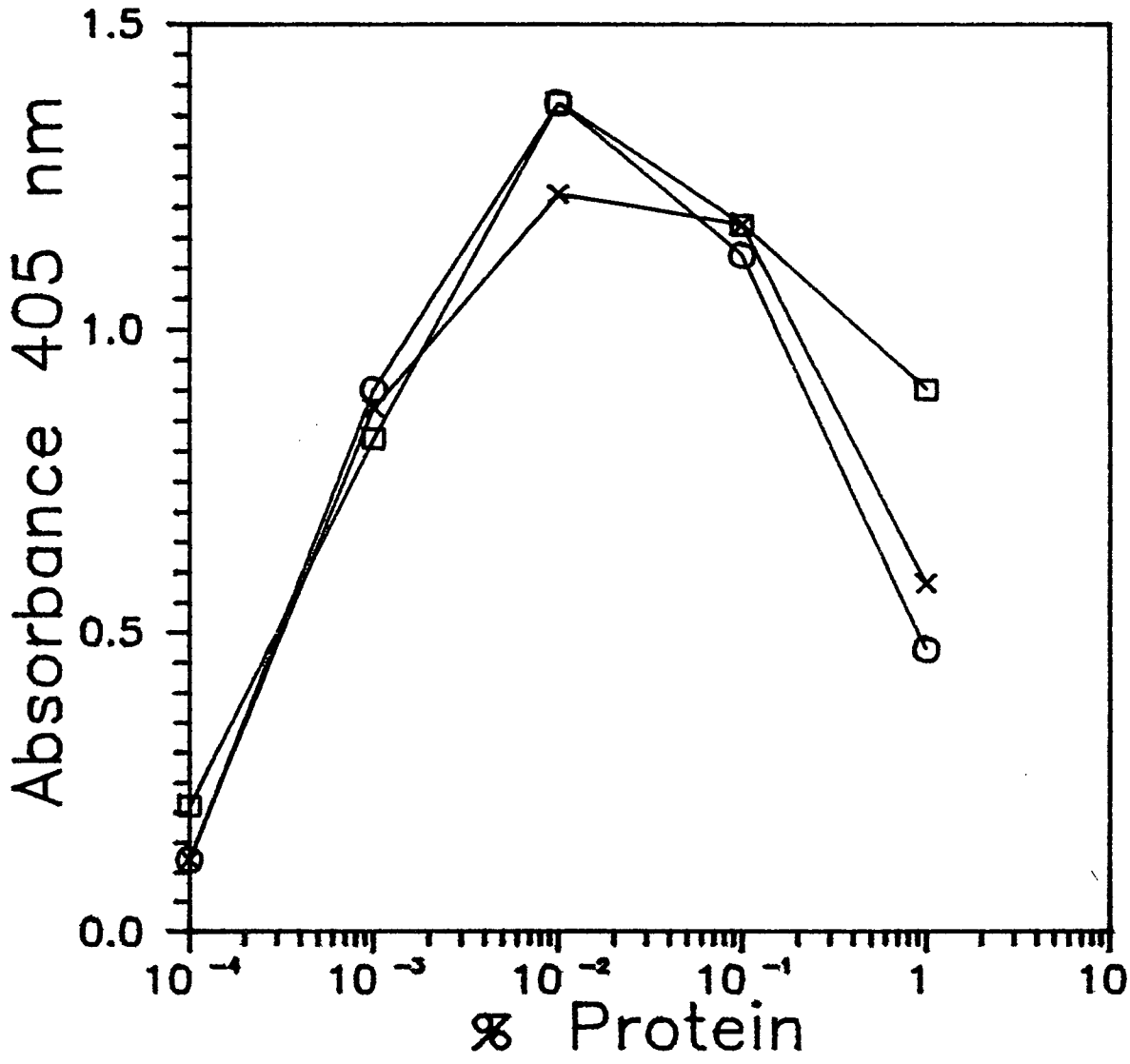

Figure 25: Antigen binding activity of IgY fractions as determined by an indirect ELISA. Fraction 1 (X); Fraction 2 (O); Fraction 3 (□).

agglutination reactions whereby in the presence of antibody excess no reaction between antigen and antibody is visible (Weir, 1988). The exact mechanism for this phenomenon is not known.

The ability of the rabbit IgG to bind to NAGase was also examined using an indirect ELISA. Figure 26 indicates that, like the chickens, rabbits immunized with NAGase produced antibodies which were capable of reacting with NAGase. Again, antibody activity peaked and then declined beyond a certain concentration.

In spite of this prozone effect, it has been shown that the antibodies produced in the rabbits and chickens were capable of reacting with NAGase and therefore have potential use in an ELISA for this enzyme.

B. Preparation of Anti-NAGase - Alkaline Phosphatase (ALP) Conjugates

Anti-NAGase antibody bound to an enzyme label was required to conduct the sandwich ELISA shown (Figure 27). Anti-NAGase chicken IgY was bound to alkaline phosphatase (ALP) using two methods. The first method involved linking free amino groups of the antibody and the enzyme by reacting with glutaraldehyde. The reaction mixture was then purified by gel filtration to remove unreacted antibody and enzyme. To determine the working dilution of the conjugate, an ELISA was performed on various dilutions of conjugate with NAGase. The conjugate had virtually no activity below a 1/10 dilution (Figure 28). The most noticable problem with the use of this

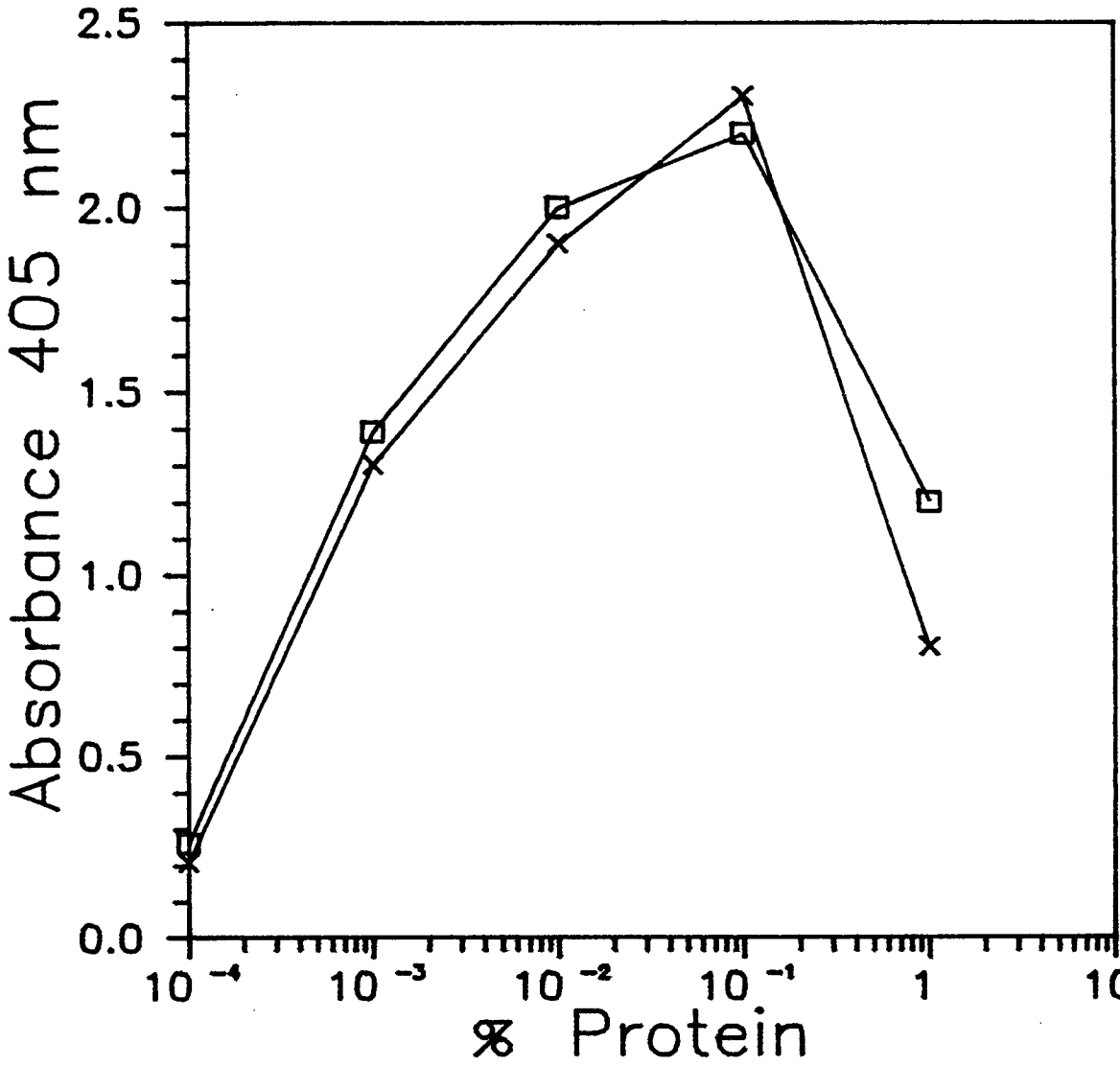

Figure 26: Antigen binding activity of isolated rabbit IgG fractions as determined by an indirect ELISA. Rabbit 1 (X); Rabbit 2 (□).

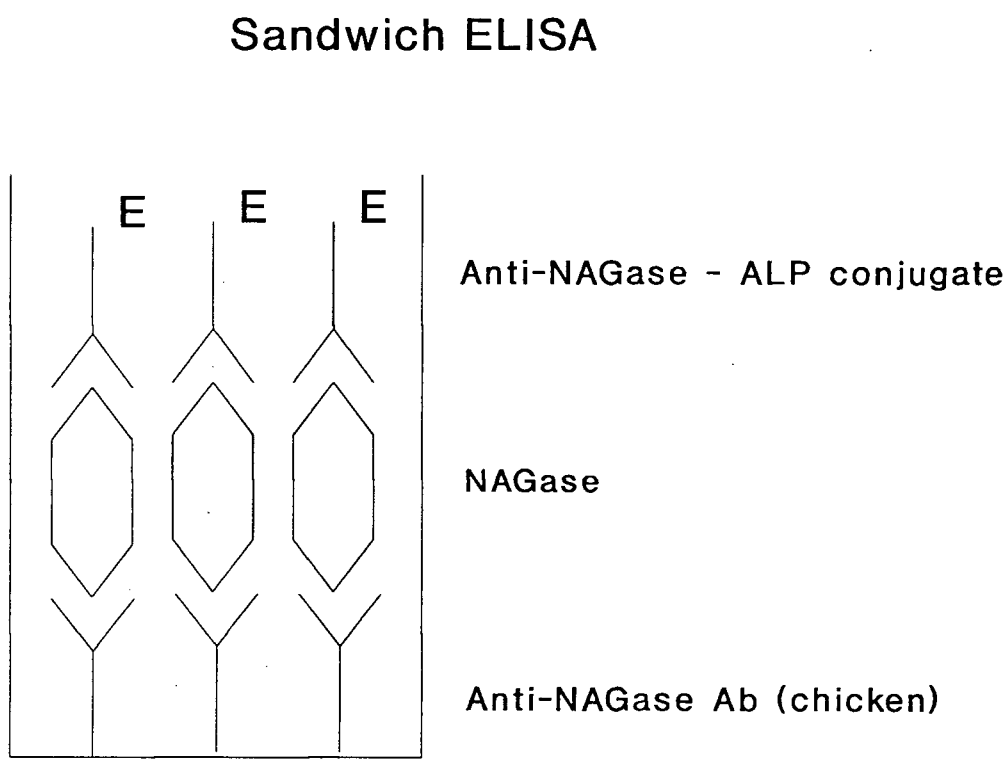

Figure 27: Diagram of the sandwich ELISA

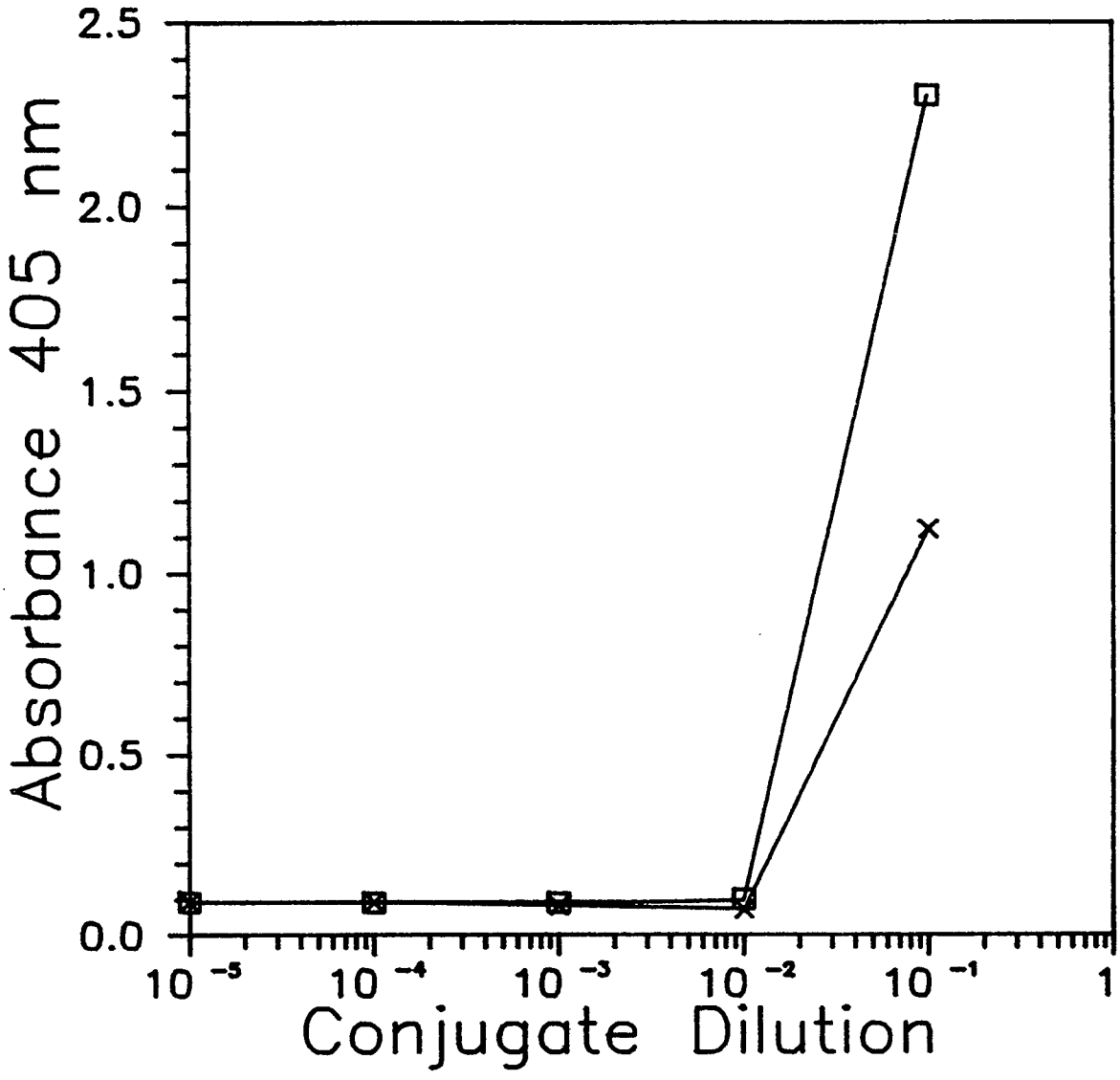

Figure 28: Determination of the working dilution of the glutaraldehyde prepared anti-NAGase - ALP conjugate. (X) with NAGase coating; (□) without NAGase coating.

conjugate was that even in the absence of NAGase (plate was still blocked), a very high absorbance was recorded when the conjugate was added. The absorbance was even greater than when NAGase was present. This indicates that the conjugate was binding non-specifically to the plate.

When compared with results from a similar ELISA using the same antibody (unconjugated), followed by addition of commercial anti-chicken IgY - ALP conjugate, non-specific binding did not occur (i.e. there was no significant absorbance reading in the absence of NAGase). This can be seen in Figure 29. This suggests that perhaps some change in the antigen binding site on the antibody molecule had taken place as a result of conjugation with ALP, resulting in an increase in non-specific binding. The non-specific binding may also be attributed to the low dilutions required for an observed antibody-antigen interaction. Masseyeff and Ferrua (1979) observed that in some instances at a high concentration of conjugate the maximal binding was the same whether antigen was present or not. Since the low reactivity and non-specific binding of the conjugate may be due to the method used to link the antibody and enzyme, a second conjugation method was chosen.

The second method employed sodium periodate as the active reagent. Sodium periodate oxidized carbohydrates to aldehydes on the enzyme which were then allowed to react with free amino groups on the antibody. The reaction mixture was purified by gel filtration. The working dilution of the

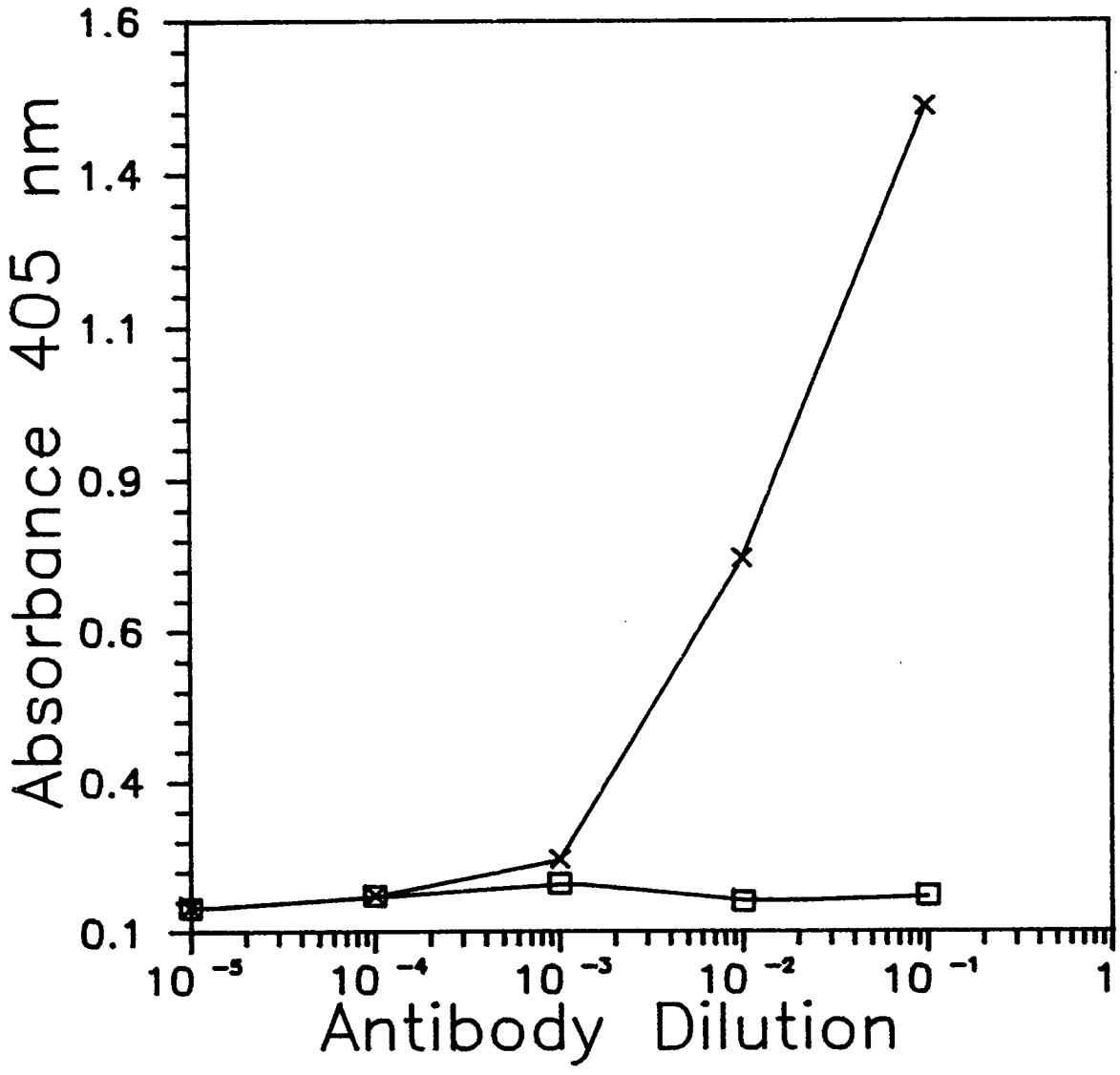

Figure 29: Determination of the working dilution of the IgY fraction used in the preparation of anti-NAGase - ALP conjugates. (X) with NAGase coating; (□) without NAGase coating.

conjugate was determined by ELISA. Results similar to those for the glutaraldehyde prepared conjugate were observed (Figure 30). While the conjugate retained some activity at as high as a 1/20 dilution, there was also a high degree of non-specific binding in the absence of NAGase.

Until the problems associated with these conjugates are solved, a sandwich ELISA for NAGase cannot be developed

C. Application of an ELISAs to detect NAGase

An attempt was made to derive standard curves for the detection of NAGase using a competitive and a double-sandwich ELISA. Application of these assays to detect NAGase in fish samples was also examined.

1. Competitive ELISA

A competitive ELISA (Figure 31) was performed on three separate days using NAGase concentrations ranging from 0 µg/ml to 20 µg/ml. Microwell plates were coated with a fixed concentration of NAGase. A fixed concentration of anti-NAGase Ab was added at the same time as the NAGase standards. A competition for binding sites occured. Anti-NAGase not bound to free NAGase was able to react with NAGase coating the plate. Anti-chicken - ALP conjugate was then added. Figure 32 depicts an average standard curve. For all three days, absorbance values increased with increasing NAGase concentration to 0.5 µg/ml then decreased with further increases in the concentration of NAGase. This relationship is not typical of a competitive ELISA. A continual decrease

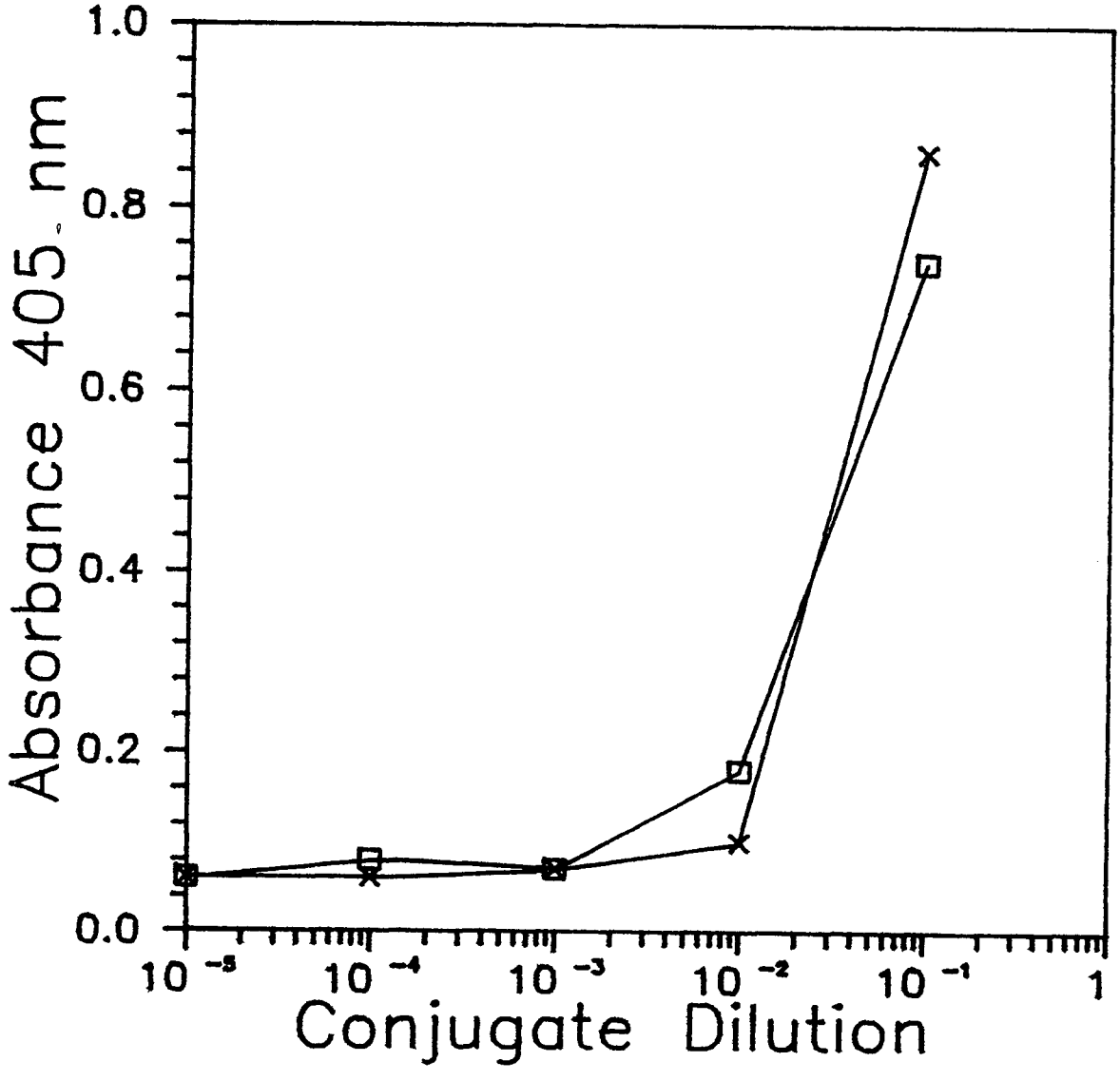

Figure 30: Determination of the working dilution of the periodate-oxidation prepared anti-NAGase - ALP conjugate. (X) with NAGase coating; (□) without NAGase coating.

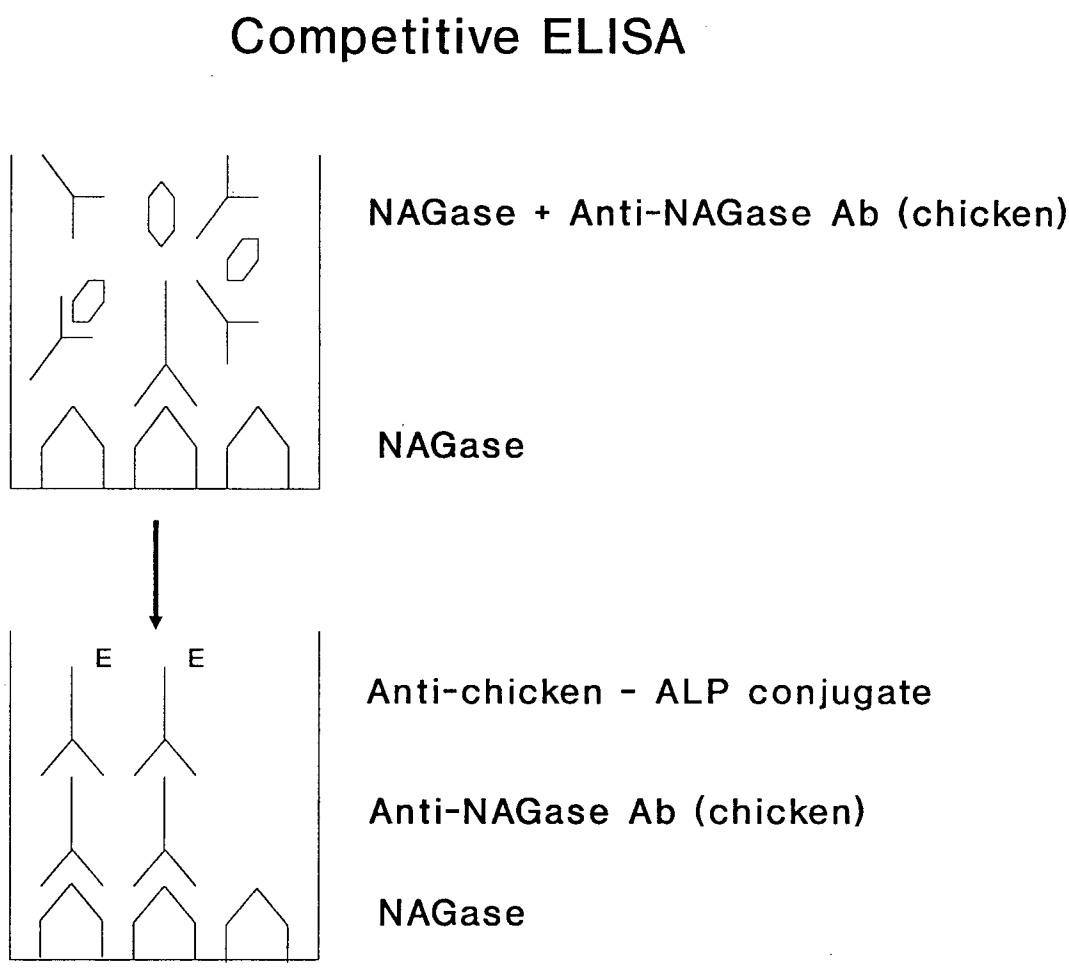

Figure 31: Diagram of the competitive ELISA.

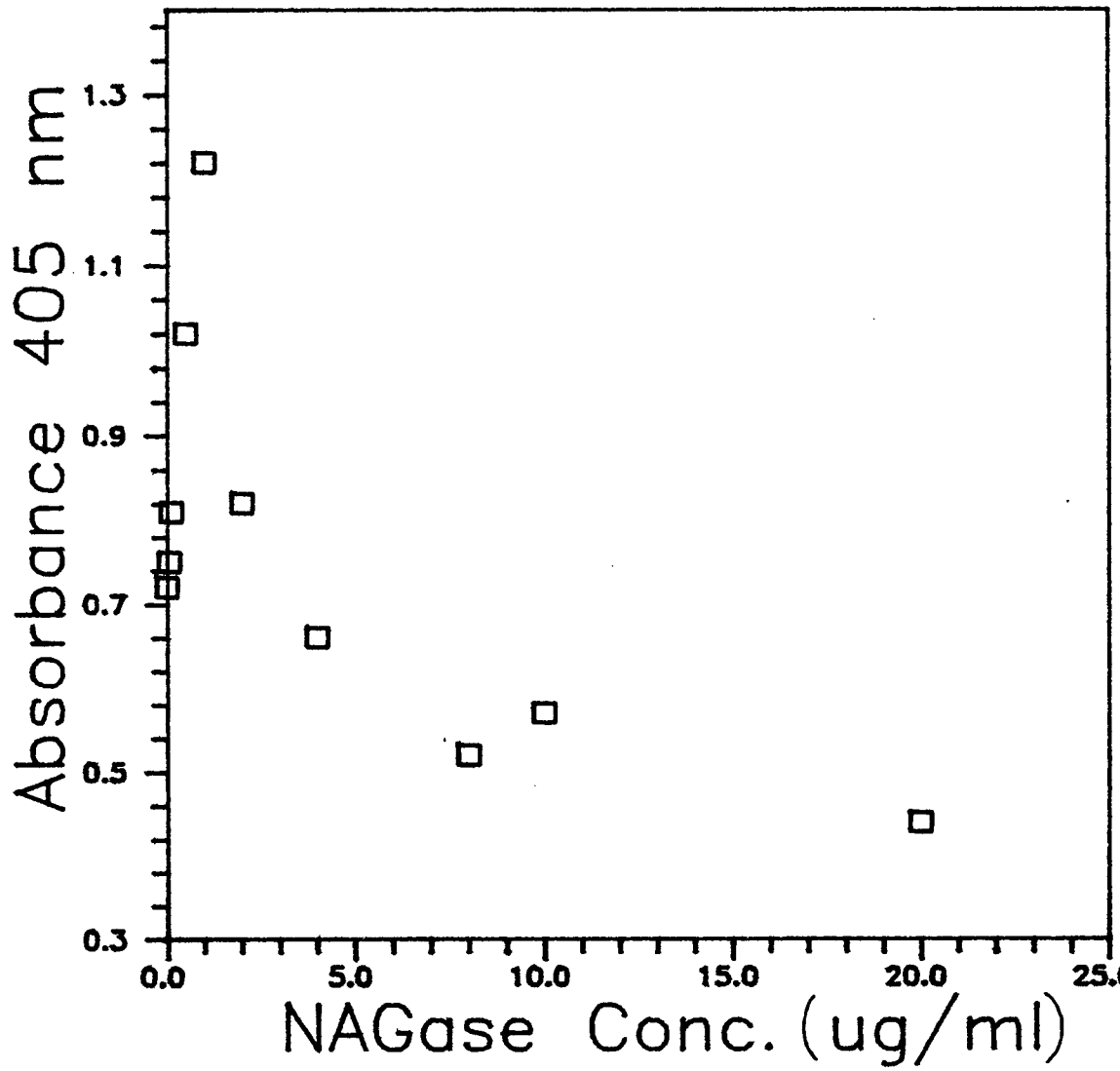

Figure 32: Relationship between NAGase concentration and absorbance at 405 nm in a competitive ELISA.

in absorbance values as the antigen concentration increases was expected (Huang et al., 1987). The data presented here suggests that below a critical NAGase concentration, increases in NAGase concentration cause an increase in binding of anti-NAGase to NAGase already coating the plate. The reason why this occured is unknown. No previous reports of such a phenomenon could be found in the literature.

Due to the nature of these results, a standard curve could not be fitted to this data. Therefore, this ELISA was not suitable for the determination of NAGase concentrations in fish samples.

2. Double-sandwich ELISA

A double-sandwich ELISA (Figure 33) was performed on three separate days using NAGase concentrations ranging from 0.031 μg/ml to 20 μg/ml. The relationship between the log of the standard concentrations and the absorbance at 405 nm was significantly linear (α=0.01) on each day. A typical standard curve is depicted in Figure 34.

In this ELISA, prepared rabbit anti-NAGase was used to coat the microwell plate and chicken anti-NAGase was used as the second antibody. The ELISA performed in this way had an average blank absorbance of 0.027 at 405 nm when no NAGase was added. When the plates were coated with chicken anti-NAGase and rabbit anti-NAGase was used as the second antibody, however, the absorbance of the blanks at 405 nm averaged 0.39. This information suggests the commercial anti-rabbit antibody - ALP conjugate cross- reacted with the

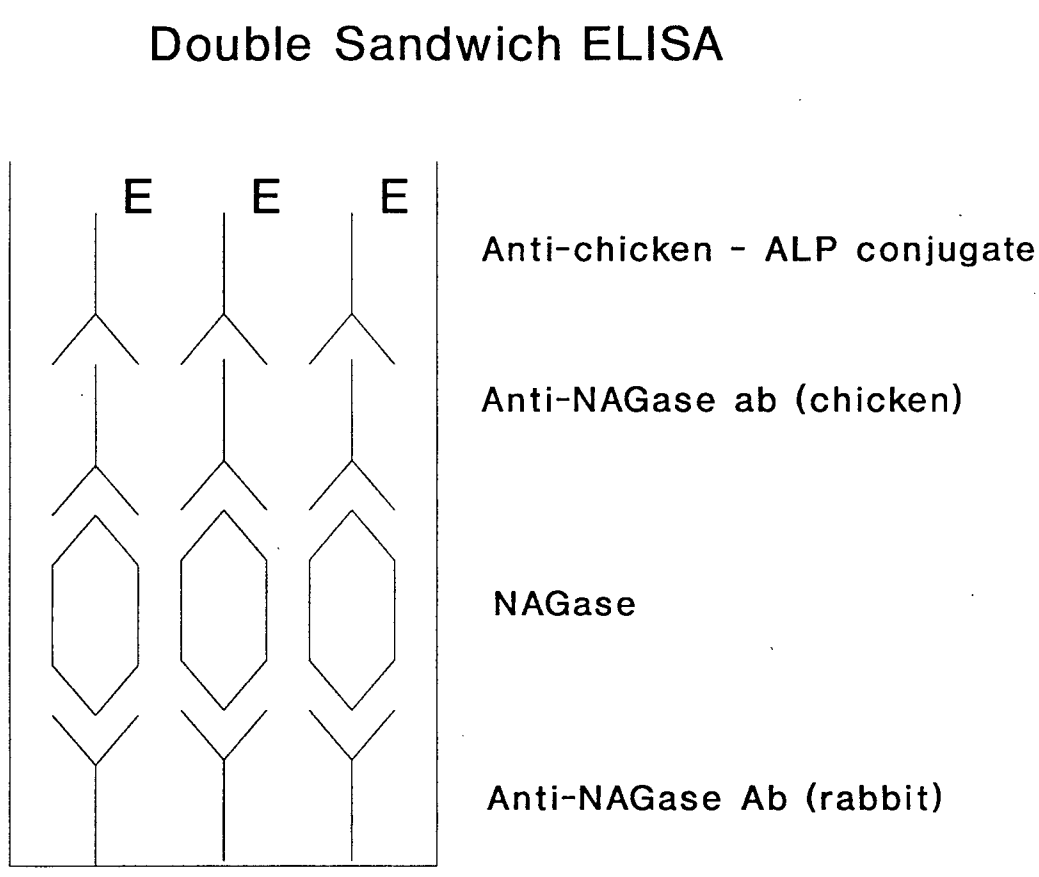

Figure 33: Diagram of the double sandwich ELISA.

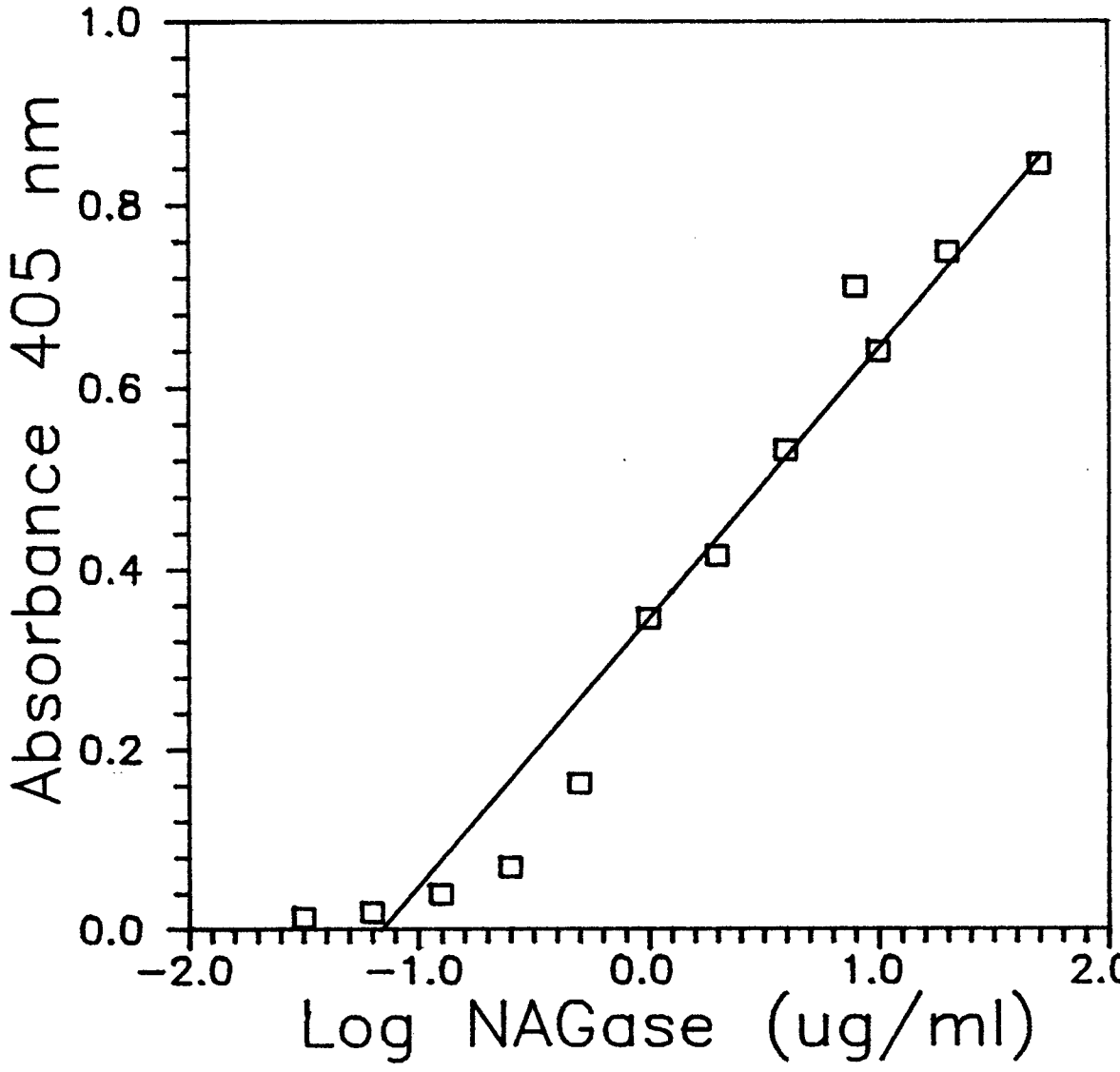

Figure 34: A typical standard curve for NAGase as determined by a double-sandwich ELISA (r^2 = 0.96; SEE = 0.066)

chicken IgY. The fact that the blank values were low when rabbit IgG was used as the coating antibody indicates that there was no cross-reaction between anti-chicken IgY - ALP conjugate and rabbit IgG or the prepared chicken IgY and the rabbit IgG. A possible explanation for these results is that the same or similar determinants to which the anti-rabbit Ig were raised also exist on the chicken IgY molecule.

D. Application of a Double-sandwich ELISA to Detect NAGase in Fish Muscle

The double-sandwich ELISA was applied to the examination of press juice (PJ) and fish extract (FE) samples from salmon muscle. The concentration of NAGase in the samples was calculated using a standard curve. Five dilutions of the PJ and FE samples were examined (no dilution, 1/2, 1/4, 1/8 and 1/16). Table VII shows the results from the 3 salmon samples examined. Results of only the first three dilutions are shown since absorbance values obtained from higher dilutions were too low to enable calculation of NAGase concentrations from the standard curve.

Similar NAGase concentrations would be expected for the different dilutions once the dilution factor had been taken into consideration. This was not the case. Instead, in all but three samples the calculated NAGase concentration increased with an increase in dilution. McCannel (1988) reported a similar occurence in her examination of diluted PJ and FE samples by an indirect ELISA. She suggested that in the indirect ELISA, other substances were coating the plate

Table VII: Levels of ß-N-Acetylglucosaminidase (NAGase) in Fresh and Frozen Salmon Samples as Determined by a Double-sandwich ELISA.

		NAGase Conc. (ug/ml)[a]		
		Sample Dilution		
Sample	Preparation	1	1/2	1/4
Salmon 1	Fresh Press Juice	0.235	0.342	0.536
Salmon 2		0.210	0.326	0.424
Salmon 3		0.251	0.370	0.360
Salmon 1	Fresh Extract	0.320	0.538	0.360
Salmon 2		0.193	0.248	0.336
Salmon 3		0.168	0.256	0.340
Salmon 1	Frozen Press Juice	0.520	0.682	0.588
Salmon 2		0.309	0.442	0.596
Salmon 3		0.240	0.342	0.544
Salmon 1	Frozen Extract	0.387	0.462	0.620
Salmon 2		0.239	0.322	0.496
Salmon 3		0.221	0.286	0.432

a. Concentrations presented were after multiplication by dilution factors.

which interfered with NAGase binding. Dilution of the fish samples therefore diluted the interfering substances and thus an increased amount of NAGase bound to the plate. A similar explanation can be offered for the results observed in this experiment. Dilution of the samples diluted other substances which possibly interfered with antibody-antigen interaction. Therefore, more NAGase bound to the anti-NAGase antibody coating the plate. The result was an observed increase in absorbance at 405 nm and the calculated NAGase concentration.

The data in Table VII was converted into ratios of NAGase concentration in the PJ fraction to the total NAGase concentration (NAGase concentration in the PJ + NAGase concentration in the FE). The ratios for the fresh and frozen samples is shown in Table VII. The expression of these data as the ratios of the NAGase concentration in the PJ to the total NAGase concentration appeared to eliminate the dilution effect (Table VIII). The ratios derived for each dilution were not significantly different as determined by analysis of variance (α=0.05). Therefore, it is reasonable to assume that any dilution of PJ or FE samples could be used to derive NAGase concentrations provided the absorbance values recorded are within the bounds of the standard curve. Rehbein et al. (1978) used a similar ratio (NAGase enzyme activity in PJ compared to total enzyme activity) to decrease variability in the data due to species of fish. Use of ratios may also eliminate variability due to other factors such as age of fish, duration of storage, etc.

Table VIII: Concentration Ratios of ß-N-Acetylglucosaminidase (NAGase) in Fresh and Frozen Salmon Samples (calculated from Table VII).

		Concentration Ratio[a]		
		Sample Dilution		
Sample	Preparation	1	1/2	1/4
Salmon 1	Fresh	0.423	0.388	0.598
Salmon 2		0.521	0.568	0.558
Salmon 3		0.599	0.591	0.514
Salmon 1	Frozen	0.573	0.596	0.487
Salmon 2		0.564	0.578	0.545
Salmon 3		0.520	0.545	0.538

a. $$\text{concentration ratio} = \frac{(\ PJ\ )}{(\ PJ + FE\ )}$$

When McCannel (1988) performed an indirect ELISA on salmon samples, she reported that NAGase concentrations decreased in the frozen samples. It was suggested that this was possibly due to partial denaturation of the native NAGase structure by freezing, resulting in reduced recognition of the NAGase by its specific antibody. Using the double sandwich ELISA, NAGase concentrations in frozen PJ samples were significantly higher as determined by analysis of variance (α=0.05), than in the same samples when fresh (Table VII). This is an expected result since it has already been established that some lysosomal enzymes including NAGase, are released during freeze/thawing of fish muscle (Rehbein et al., 1978). Therefore, the concentration of NAGase is expected to be higher in the frozen PJ samples.

Analysis of variance was performed on the concentration ratios for the fresh samples versus samples frozen for one week then thawed. The analysis showed that there was no significant difference (α=0.05) between the two sets of ratios and therefore the two treatments. This indicates that while the levels of NAGase increased in the frozen PJ samples, so also did the total NAGase levels. Total NAGase levels would be expected to remain the same for both the fresh and the frozen treatments. One explanation for the difference in total NAGase levels may be that the conditions used for the preparation of fish extract were not severe enough for the disruption of lysosomes and release of NAGase in the fresh fish. Lysosomes in the frozen fish muscle, on

the other hand, may have suffered freeze damage. As a result they were more susceptible to lysosome disruption and NAGase release. The result was that the NAGase levels in the frozen FE were higher than in the fresh FE. Therefore, the calculated total NAGase levels were higher in the frozen samples.

In order to use this ELISA to differentiate between frozen/thawed and fresh fish, a method is needed for fish extract preparation which would result in consistant total NAGase concentrations in fresh and frozen/thawed samples. Rehbein et al. (1978) used a fish extract preparation method similar to the one described in this experiment. However, Triton X-100 was added prior to homogenization. This detergent probably aided in membrane disruption and solubilization of membrane bound proteins. The result was a more reliable estimation of, in this case, total NAGase enzyme activity. By comparing the NAGase activity in the press juice as a % of the total activity, these researchers were able to detect significant differences between fresh fish fillets and fillets frozen for 1 day at -26 to -29 ^{o}C. Triton X-100 was not added in the present study because it was thought that it may affect antigen-antibody binding. Experiments to determine if this were true, however, were not performed.

To effectively use this ELISA, one has to establish that the differences in NAGase concentration ratios are solely due to freezing. Experiments to test this hyothesis were not

done in this thesis. Rehbein et al. (1978) have already reported that during autolysis and bacterial spoilage of fish muscle, enzymes had been released as indicated by activity ratios for spoiled fish which were nearly as high as in the frozen/thawed fillets. However, for NAGase, the activity ratios increased after 10 days of storage of fish on ice. The fish were not sellable at that point based on organoleptic and chemical tests (Rehbein et al., 1978). Therefore, the estimation of NAGase levels in fish muscle may still be a useful indicator of frozen/thawed fish muscle.

E. Conclusion

Polyclonal antibodies specific for NAGase were successfully derived from both chickens and rabbits. However, chicken IgY - ALP conjugates exhibited low immunoreactivity with the antigen as well as a high degree of non-specific binding when prepared by reaction with glutaraldehyde or by periodate oxidation. Therefore, these conjugates were considered unsuitable reagents for use in a sandwich ELISA. Problems were also encountered in the development of a competitive ELISA for NAGase as seen by the atypical standard curve produced by this assay.

Development of a double-sandwich ELISA for NAGase was successful. However, when this ELISA was applied to the detection of this enzyme in fish muscle samples, the information obtained was inconclusive. While increases in the levels of this enzyme were observed in frozen PJ samples, concentration ratios remained the same. Changes in the

procedure for FE preparation, such as the addition of detergents, may improve the results. At the same time, however, it should be determined if these detergents have an effect on antigen-antibody interaction. If this is the case, alternative methods should be explored. With this modification it is expected that the double-sandwich ELISA for NAGase would be an effective method for distinguishing frozen/thawed from fresh fish muscle.

BIBLIOGRAPHY

Altschuh, D., Hennache, G. and Van Regenmortel, M.H.V. 1984. Determination of IgG and IgM levels in serum by rocket immunoelectrophoresis using yolk antibodies from immunized chickens. J. Immunol. Methods 69:1.

Back, E., Mollby, R., Kaijser, B., Stintzing, G. Wadstrom, T. and Habte, D. 1980. Enterotoxigenic Escherichia coli and other gram-negative bacteria of infantile diarrhea: surface antigens, hemagglutinins, colonization factor antigen and loss of enterotoxigenicity. J. Infect. Dis. 142:318.

Bankert, R.B., Jou, Y. and Mayers, G.L. 1984. Monoclonal antibodies/myelomas and hybridomas. In: Molecular Immunology. Atassi, M.Z., Van Oss, C.J. and Absolom, D.R. eds. Marcel Dekker, Inc., New York, NY, pp. 175-200.

Bellanti, J.A. and Kadlec, J.V. 1985. Introduction to immunology. In: Immunology: Basic Processes. Bellanti, J.A. ed. W.B. Saunders Co., Toronto, ON, pp. 1-15.

Bellanti, J.A. and Rocklin, R.E. 1985. Cell-mediated immune reactions. In: Immunology: Basic Processes. Bellanti, J.A. ed. W.B. Saunders Co., Toronto, ON, pp. 176-188.

Bernier, G.M. 1985. Antibody and immunoglobulins: structure and function. In: Immunology: Basic Processes. Bellanti, J.A. ed. W.B. Saunders Co., Toronto, ON, pp. 89-105.

Bigazzi, P.E. and Tilton, R.C. 1980. Immunofluorescence and immunoenzyme procedures. In: Methods in Immunodiagnostics. Rose, N.R. and Bigazzi, P.E. eds. John Wiley and Sons, Toronto, ON, pp. 161-202.

Black, R.E., Hug, I., Merson, M.H., Alim, A.R.M.A. and Yunus, M.D. 1981. Incidence and severity of rotavirus and E.coli diarrhea in rural Bangladesh. Implications for vaccine development. Lancet 1:141.

Borrebaeck, C. and Glad, C. 1989. Cross-reactivity of mouse monoclonal antibodies produced by in vitro or in vivo immunization. Immunol. Lett. 20:255.

Bosch, A.M.G., Stevens, W., Schuurs, A., Schonherr, O., and Roelofs, H. 1982. Characteristics of monoclonal antibodies against human chorionic gonadotrophin. Prot. Biol. Fluids 29:837.

Brodeur, B.R. and Tsang, P.S. 1986. High yield of monoclonal antibody production in ascites. J. Immunol. Methods 86: 239.

Bruck, C., Drebin, J.A., Glineur, C. and Portetelle, D. 1986. Purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affi-Gel Blue chromatography. Methods in Enzymol. 121:587.

Bruggeman, M., Radbruch, A. and Rajewsky, K. 1982. Immunoglobulin V region variants in hybridoma cells. I. Isolation of a variant with altered idiotypic and antigen binding specificity. EMBO J. 1:629.

Burchiel, S. 1986. Purification and analysis of Mab by analysis of high performance liquid chromatography. Methods in Enzymol. 121:596.

Burchiel, S.W., Billman, R. and Alber, T.R. 1984. Rapid and efficient purification of mouse monoclonal antibodies from ascites fluid using high performance liquid chromatography. J. Immunol. Methods 69:33.

Burger, D., Ramus, M.A. and Schapiro, M. 1985. Antibodies to human plasma kallikrein from egg yolks of an immunized hen: preparation and characterization. Thrombosis Res. 40:283.

Burnet, F.M. 1957. A modification of Jerne's theory of antibody production using the concept of clonal selection. Austr. J. Sci. 20:67.

Butman, B.T., Plank, M.C., Durham, R.J. and Mattingly, J.A. 1988. Monoclonal antibodies which identify a genus-specific Listeria antigen. Appl. Environ. Microbiol. 54:1564.

Calvanico, N.J. 1984. Structure and function of immunoglobulins. In: Molecular Immunology. Atassi, M.Z., Van Oss, C.J. and Absolom, D.R. eds. Marcel Dekker, New York, NY, pp. 141-174.

Cleary, T.G., Mathewson, J.J., Faris, E. and Pickering, L.K. 1985. Shiga-like cytotoxin production by enteropathogenic Escherichia coli serogroups. Infect. Immun. 47:335.

Clezardin, P., Hunter, N.R., MacGregor, I.R., MacGregor, J.L. Pepper, P.S. and Dawes, J. 1986. Tandem purification of mouse IgM monoclonal antibodies produced in vitro using anion-exchange and gel fast protein liquid chromatography. J. Chromatog. 358:209.

Cotton, R.G.H., Secher, D.S. and Milstein, C. 1973. Somatic mutation and the origin of antibody diversity. Clonal variability of the immunoglobulin produced by MOPC 21 cells in culture. Eur. J. Immunol. 3:135.

Cravioto, A., Gross, R.J., Scotland, S.M. and Rowe, B. 1979. An adhesive factor found in strains of Escherichia coli belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. 3:95.

Crimes, A.A., Hitchcock, C.H.S. and Wood, R. 1984. Determination of soya protein in meat products by an enzyme-linked immunosorbent assay procedure: collaborative study J. Assoc. Pub. Analysts 22:59.

Crow, E.L., Davis, F.A. and Maxfield, M.W. 1978. Statistics. Coles Publishing Co., Toronto, ON, p. 159.

de Savigny, D. and Voller, A. 1980. The communication of ELISA data from laboratory to clinician. J. Immunoassay 1:105.

Dunn, S.D. 1986. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins in western blots by monoclonal antibodies. Anal. Biochem. 157:144.

Echeverria, P., Seriwatana, J. Sethabutr, O. and Taylor, D.N. 1985. DNA hybridization in the diagnosis of bacterial diarrhea. Clin. Lab. Med. 5:447.

Eckner, K.F., Flowers, R.S., Robison, B.J., Mattingly, J.A., Gabis, D.A. and Silliker, J.H. 1987. Comparison of Salmonella Bio-Enzabead® immunoassay method and conventional culture procedure for detection of salmonella in foods. J. Food Prot. 50:379.

Editorial, 1983. Mechanisms of enteropathogenic E.coli diarrhea. Lancet 1:1254.

Edwin, C., Tatini, S.R., Strobel, R.S. and Maheswaran, S.K. 1984. Production of monoclonal antibodies to staphylococcal enterotoxin A. Appl. Environ. Microbiol. 48:1171.

Engvall, E. and Carlsson, H.E. 1976. Enzyme-linked immunosorbent assay, ELISA. In: First International Symposium on Immunoenzymatic Techniques INSERM Symposium North-Holland Publishing Co., Amsterdam, The Netherlands, pp. 136-148.

Engvall, E. and Perlmann, P. 1971. Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. Immunochem. 8:871.

Engvall, E. and Perlmann, P. 1972. Enzyme-linked immunosorbent assay, ELISA, III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol. 109:129.

Eshhar, Z. 1985. Monoclonal antibody strategy and techniques. In: Hybridoma Technology in the Biosciences. Springer, T.A. ed. Plenum Press, New York, NY, pp. 3-41.

Fahey, J.L. and Terry, E.W. 1969. Ion exchange chromatography and gel filtration. In: Handbook of Experimental Immunology. Weir, D.M. ed. Blackwell Scientific Publications, Boston, MA, pp. 19-44.

Farber, J.M. and Spiers, J.I. 1987. Monoclonal antibodies directed against the flagellar antigens of Listeria species and their potential in EIA-based methods. J. Food Prot. 50:479.

Franek, F. 1986. Purification of IgG monoclonal antibodies from ascitic fluid based on rivanol precipitation. Methods in Enzymol. 121:631.

Galfre, G. and Milstein, C. 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods in Enzym. 73:3.

Galfre, G., Howe, S.C. and Milstein, C. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 266:550.

Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. 1977. Methods in Immunology 3rd ed. W.A. Benjamin Inc., Don Mills, ON, pp. 218-219.

Ghebrehiwet, B. 1986. Production and characterization of a murine monoclonal IgM antibody to human C1q receptor (C1qR). J. Immunol. 137:618.

Gooi, H.C. and Feizi, T. 1982. Natural antibodies as contaminants of hybridoma products. Biochem. Biophys. Res. commun. 106:539.

Guerrant, R.L., Kirchhoff, L.V., Shields, D.S., Nations, M. K., Leslie, J., de Sousa, M.A., Araujo, J.G., Correia, L.L., Sauer, K.T., McClellant, K.E., Trowbridge, F.L. and Hughes, J.M. 1983. Prospective study of diarrheal illness in north-eastern Brazil: patterns of disease, nutritional impact, etiologies and risk factors. J. Infect. Dis. 148:986.

Guidry, A.J., Srikumaran, S. and Goldsby, A. 1986. Production and characterization of bovine X murine hybridomas. Methods in Enzymol. 121:244.

Guttman, R.D., Bach, F.H., Bach, M.K., Claman, H.N., David, J.R., Jeannet, M., Lindquist, R.R., McKhan, C.F., Papermaster, D. and Schwartz, R.S. 1981. Immunology Upjohn Co., Kalamazoo, MI, pp. 7-20.

Hamm, R. and Masic, D.1971. Einflub des Gefrierens und Auftauens von karpfen auf die subzellulare verteilung der aspartat aminotransferase im skeletmuskel. Arch. Fisch-Wiss. 22:121.

Hofstra, H. and Dankert, J. 1980. Antigenic cross-reactivity of major outer membrane proteins in Enterobacteriaceae species. J. Gen. Microbiol. 111:293.

Horejsi, V. and Hilgert, I. 1986. Simple polyacrylamide gel electrophoresis in continuous carbonate buffer system suitable for the analysis of ascitic fluids of hybridoma bearing mice. J. Immunol. Methods 86:103.

Huang, S.C., Miki, K., Hirano, K., Hayashy, Y., Furihata, C., Shimizu, A., Ichinose, M., Oka, H., Matsushima, T. and Takahashi, K. 1987. Enzyme-linked immunosorbent assay of serum pepsinogen I. Clin. Chim. Acta 162:85.

Kaminogawa, S., Hattori, M., Ando, O., Kurisaki, J.I. and Yamauchi, K. 1987. Preparation of monoclonal antibody against bovine β-lactoglobulin (a potent allergen in cows' milk allergy) and its unique binding affinity. Agric. Biol. Chem. 51:797.

Kannangara, T., Wieczorek, A. and Lavender, D.P. 1989. Immunoaffinity columns for isolation of abscisic acid in conifer seedlings. Physiol. Plant. 75:369.

Knutton, S. and Pasternak, C.A. 1979. The mechanism of cell-cell fusion. Trends Biochem. Sci. 4:220.

Knutton, S., Baldwin, T., Williams, P.H. and McNeish, A.S. 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: Basis of a new diagnostic test for enteropathogenic and enterohemorrhagic E.coli. Infect. Immun. 57:1290.

Kohler, G. 1986. Derivation and diversification of monoclonal antibodies. Science 233:1281.

Kohler, G. and Milstein, C. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256:495

Kurstak, E. 1986. Enzyme Immunodiagnostics. Academic Press Inc., Toronto, ON, pp. 14-32.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680.

Lambert, D. 1988. Enterobacteriaceae: composition, structure and function of the cell envelope. J. Appl. Bacter. Symposium Supplement 21S-34S.

Lane, R.D., Crissman, R.S. and Ginn, S. 1986. High efficiency fusion procedure for producing monoclonal antibodies against weak immunogens. Methods in Enzymol. 121:183.

Leslie, G.A. and Clem, L.W. 1969. Phylogeny of immunoglobulin structure and function III. Immunoglobulins of the chicken. J. Exp. Med. 130:1337.

Levine, M.M. 1987. Eschericia coli that cause diarrhea: enterotoxigenic, enteropathogeic, enteroinvasive, enterohemorrhagic and enteroadherent. J. Infect. Dis. 155:377.

Levine, M.M., Nataro, J.P., Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.L. and O'Brien, A.D. 1985. The diarrheal response of humans to some classical Escherichia coli is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. 152:550.

Littlefield, J.W. 1964. Selection of hyrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145:709.

Lopez-vidal, Y., Klemm, P. and Svennerholm, A.M. 1988. Monoclonal antibodies against different epitopes on colonization factor antigen I of enterotoxin producing Escherichia coli. J. Clin. Mibrobiol. 26:1967.

Marler, E., Nelson, C.A. and Tanford, C. 1964. The polypeptide chains of rabbit γ-globulin and its papain cleaved fragments. Biochem. 3:279.

Masseyeff, R. and Ferrua, B. 1979. Problems raised by the evaluation of conjugates used in enzyme immunoassays. In: Immunoenzymatic Assay Techniques. Malvano, R. ed. Martinus Nijhoff Publishers, Boston, MA, pp. 28-44.

May, K., Senior, S.J. and Porter, P. 1984. The structure and synthesis of immunoglobulins. In: Immunoglobulin Aspects of Reproduction in Mammals. Crighton D.B. ed. Butterworths, Toronto, ON, pp. 13-26.

McCannel, A.M. 1988. Separation of immunoglobulins from egg yolk using metal chelate interaction chromatography and ion exchange chromatography. MSc. Thesis, University of British Columbia, Vancouver, BC.

Milford-Ward, A. 1981. Immunoprecipitation in the evaluation of the proteins in plasma and body fluids. In: Techniques in Clinical Immunology. Thompson, R.A. ed. Blackwell Scientific Publications, Boston, MA, pp. 1-24.

Monroe, D. 1984. Enzyme immunoassay. Anal. chem. 56:920A.

Moriarty, K.J. and Turnberg, L.A. 1986. Bacterial toxins and diarrhoea. Clin. Gastroent. 15:529.

Nataro, J.P., Baldini, M.M., Kaper, J.B., Black, R.E., Bravo, N. and Levine, M.M. 1985. Detection of an adherence factor of enteropathogenic E.coli with a DNA probe. J. Infect. Dis. 152:560.

Notermans, S., Heuvelman, C.J., Egmond, H.P., van Paulsch, W. E. and Besling, J.R., 1986. Detection of mold in food by enzyme-linked immunosorbent assay. J. Food Prot. 49:786.

O'Brien, A.D., LaVeck, G.D., Thompson, M.R. and Formal, S.B. 1982. Production of Shigella dysenteriae type 1-like cytotoxin by Escherichia coli. J. Infect. Dis. 146:763.

O'Brien, A.D., Lively, T.A., Chen, M.E., Rothman, S.W. and Formal, S.B. 1983. Escherichia coli 157:H7 strains associated with hemorrhagic colitis in the United States produce a Shigella dysenteriae 1(shiga) - like cytotoxin. Lancet 1:702.

Olsson, J., Kronstrom, H., Cambon-de Mouzon, A., Honsik, C., Brodin, T. and Jakobsen, B. 1983. Antibody producing human - human hybridomas. I. Technical aspects. J. Immunol. Methods 61:17.

Patterson, R.L.S. and Jones, S.J. 1983. Species identification of meat in raw, unheated meat products. In: Immunoassays in Food Analysis. Morris, B.A. and Clifford, M.N. eds. Elsevier Applied Science Publishers, New York, NY, pp. 87-94.

Pesce, A.J., Ford, D.J. and Gaizutis, P. 1978. Qualitative and quantitative aspects of immunoassays. Scand. J. Immunol. 8(Suppl 7):1.

Polson, A., Von Wechmar, B. and Van Regenmortel, M.H. 1980. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol. Commun. 9:475.

Preud'homme, J.L., Birshtein, B.K. and Scharff, M.D. 1975. Variants of a mouse myeloma cell line which synthesize immunoglobulin heavy chains having an altered serotype. Proc. Natl. Acad. Sci. USA. 72:1427.

Quaranta, H.O. and Perez, S.S. 1983. chemical methods for measuring changes in freeze stored fish: A review. Food Chem. 11:79.

Ram, B.P., Hart, L.P., Cole, R.J. and Pestka, J.J. 1986. Application of ELISA to retail survey of aflatoxin B_1 in peanut butter. J. Food Prot. 49:792.

Rehbein, H. 1979. Development of an enzymatic method to differentiate fresh and sea-frozen and thawed fish fillets I. comparison of the applicability of some enzymes of fish muscle. Z. Lebensm. Unters. Forsch. 169:263.

Rehbein, H., Kress, G. and Schreiber, W. 1978. An enzymatic method for differentiating thawed and fresh fish fillets J. Sci. Fd. Agric. 29:1076.

Rennels, M.B. and Levine, M.M. 1986. Classical bacterial diarrhea: perspectives and update -- Salmonella, Shigella, Escherichia coli, Aeromonas and Plesiomonas. Ped. Infect. Dis. 5:591.

Robins-Browne, R.M. 1987. Traditional enteropathogenic E.coli of infantile diarrhea. Rev. Infect. Dis. 9:28.

Robison, B.J., Pretzman, C.I. and Mattingly, J.A. 1983. Enzyme immunoassay in which a myeloma protein is used for detection of salmonellae. Appl. Environ. Microbiol. 45:1816.

Roitt, I. 1988. Essential Immunology. Blackwell Scientific Publications, Boston, MA, pp. 2-29.

Rose, M.E. and Orlans, E. 1981. Immunoglobulins in the egg, embryo and young chick. Dev. Comp. Immunol. 5:15.

Rothbaum, R.J., Partin, J.C., Saalfield, K. and McAdams, A.J. 1983. An ultra-structural study of enteropathogenic E.coli infection in human infants. Ultrastruc. Path. 4:291.

Ruitenberg, E.J., Van Knapen, F. and Elgersma, A. 1983. Surveillance in swine by immunodiagnostic methods. In: Trichinella and Trichinosis. Campbell, W.C. ed. Plenum Press, New York, NY, pp. 529-550.

Saunders, G.C. and Bartlett, M.L. 1977. Double antibody solid-phase enzyme immunoassay for the detection of staphylococcal enterotoxin A. Appl. Environ. Microbiol. 34:518.

Saunders, G.C. and Clinard, E.H. 1984. Rapid micromethod of screening for antibody to disease agents using the indirect enzyme-labelled antibody test. J. Clin. Microb. 3:604.

Shahbaz, A. and Rubina, A. 1986. Infantile diarrhea due to enteropathogenic E.coli. JPMA 36:14.

Shibier, O., Hampton, S.M. and Marks, V. 1988. An immunization protocol which enhances the frequency of antigen-specific monoclonal antibody production. J. Immunol. Methods 114:49.

Shimizu, M., Fitzsimmons, R.C. and Nakai, S. 1988a. Anti-E.coli immunoglobulin isolated from egg yolk of immunized chicken as a potential food ingredient. J. Food Sci. 53:1360.

Shimizu, M. 1988b. Personal communication.

Shone, C., Wilton-Smith, P., Appleton, N., Hambleton, P., Modi, N., Gatley, S. and Melling, J. 1985. Monoclonal antibody-based immunoassay for type A Clostridium botulinum toxin is comparable to mouse bioassay. Appl. Environ. Microbiol. 50:63.

Sirvaganian, R.P., Fox, P.C. and Berenstein, E.H. 1983. Methods of enhancing the frequency of antigen specific hybridomas. Methods in Enzymol. 92:17.

Skerritt, J.H. 1985. A sensitive monoclonal antibody based test for gluten detection: studies with cooked or processed foods. J. Sci. Fd. Agric. 36:987.

Skerritt, J.H., Smith, R.A., Wrigley, C.W. and Underwood, P.A. 1984. Monoclonal antibodies to gliadin proteins used to examine cereal grain protein homologies. J. Cereal Sci. 2:215.

Spiegelberg, H.L. 1974. Biological activities of immunoglobulins of different classes and subclasses. Advan. Immunol. 19:259.

Spira, G., Bargellesi, A., Teillaud, J.L. and Scharff, M.D. 1984. The identification of monoclonal class switch variants by sib selection and an ELISA assay. J. Immunol. Methods 74:307.

Stahli, C., Staehelin, Th. and Miggiano, V. 1983. Spleen cell analysis and optimal immunization for high-frequency production of specific hybridomas. Methods in Enzymol. 92:26.

Stepaniak, L., Birkeland, S.E., Vagias, G. and Sorhaug, T. 1987. Enzyme-linked immunosorbent assay (ELISA) for monitoring the production of heat stable proteinases and lipase from Pseudomonas (in milk). Milchwissenschaft 42:168.

Svennerholm, A.M., Wikstrom, M., Lindholm, L. and Holmgren, J. 1986. Monoclonal antibodies and immunodetection methods for Vibrio cholerae and Escherichia coli enterotoxins. In: Monoclonal Antibodies Against Bacteria III. Macario, A.J.L. and Conway de Macario, E. eds. Academic Press Inc., Toronto, ON, pp. 77-97.

Taggart, R.T. and Samloff, I.M. 1983. Stable antibody-producing murine hybridomas. Science 219:1228.

Towbin, H. and Gordon, J. 1984. Immunoblotting and dot immunobinding. current status and outlook. J. Immunol. Methods 72:313.

Tussen, P. and Kurstak, E. 1984. Highly efficient and simple methods for the preparation of peroxidase and active peroxidase-antibody conjugates for enzyme immunoassays. Anal. Biochem. 136:451.

Vaag, P. 1985. Enzyme-linked immunosorbent assay for quality control in beer. Eur. Brew. Conv. Proc. Congr. 20:547.

Vandekerckhove, P., Demeyer, D. and Henderickx, H. 1972. Evaluation of a method to differentiate between nonfrozen and frozen and thawed meat. J. Food Sci. 37:636.

Vienken, J. and Zimmerman, U. 1985. An improved electrofusion technique for production of mouse hybridoma cells. FEBS Lett. 182:278.

Voller, A. 1980. Heterogeneous enzyme-immunoassays and their applications. In: Enzyme Immunoassay. Maggio, E.T. ed. CRC Press Inc., Boca Raton, FL, pp. 181-196.

Voller, A., Bartlett, A. and Bidwell, D.E. 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clin. Pathol. 31:507.

Waldmann, M.C.H. 1986. Production of murine monoclonal antibodies. In: Monoclonal Antibodies. Beverly, P.C.L. ed. Churchill Livingstone, New York, NY, pp. 1-20.

Weber, K. and Osborn, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-page. J. Biol. Chem. 244:4406.

Weir, D.M. 1988. Immunology: Student Notes. Churchill Livingstone, New York, NY,

Westerwoudt, R. 1986. Factors affecting production of Mab. Methods in Enzymol. 121:3.

Wieczorek, A. 1988. Personal communication.

Wieczorek, A. 1989. Personal communication.

Williams, D.G. 1984. Comparison of three conjugation procedures for the formation of tracers for use in enzyme immunoassays. J. Immunol. Methods 72:261.

Wnek, A.P., Strouse, R.J. and McClane, B.A. 1985. Production and characterization of monoclonal antibodies against Clostridium perfringens type A enterotoxin. Infect. Immun. 50:442.

World Health Organization. 1987a. Weekly Epidemiological Record. 62:85.

World Health Organization. 1987b. Weekly Epidemiological Record. 62:85.

Yelton, D.E., Thammana, P., Desaymard, C. and Scharff, M.D. 1981. Monoclonal antibodies: The production of tailor-made serological reagents. In: Monoclonal Antibodies in Drug Development. August, J.T. ed. Proceedings of the First John Jacob Abel Symposium on Drug Development. pp. 3-14.

Yoshioka, K. 1983a. Differentiation of freeze-thawed fish from fresh fish by the determination of a hematocrit value. Bull. Japn. Soc. Sci. Fish. 49:149.

Yoshioka, K. 1983b. Differentiation of freeze-thawed fish from fresh fish by the examination of medulla of crystalline lens. Bull. Japn. Soci. Sc. Fish. 49:151.

Yoshioka, K. 1988. Personal communication.

Yoshioka, K. and Kitamikado, M. 1988. Differentiation of freeze-thawed fish fillet from fresh fish fillet by the examination of erythrocytes. Nippon Suisan Gakkaishi 54:1221.

Young, L.S. 1984. Monoclonal antibodies: technology and application to gram-negative infections. Infection 12:303.

Zola, H. and Brooks, D. 1982. Techniques for the production and characterization of monoclonal hybridoma antibodies. In: Monoclonal Hybridoma Antibodies: Techniques and Applications. Hurrell, J.G.R. ed. CRC Press Inc., Boca Raton, FL, pp. 1-58.