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DEVELOPMENT AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY BASED ELISA FOR MONITORING LIPID OXIDATION

By

Tamara L. Zielinski

A THESIS

Submitted to
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ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF A MONOCLONAL BASED ELISA FOR MONITORING LIPID OXIDATION

By

Tamara L. Zielinski

There is interest in developing rapid methods to monitor lipid oxidation as chromatographic methods are tedious and require sophisticated equipment. Objectives of this study were to produce and characterize monoclonal antibodies to hexanal, a common index of lipid oxidation in food, then devise and verify an indirect competitive enzyme linked immunosorbent assay (IC-ELISA) to quantify hexanal. Monoclonal antibodies against hexanal modified protein were produced and an IC-ELISA with a working range of 1-50 ng hexanal/mL was devised. Antibodies cross-reacted 37.9%, 76.6% and 45.0%, respectively, with pentanal, heptanal and 2-*t*-hexenal protein conjugates. Antibodies reacted strongly with hexanal-aminocaproic acid conjugates, but did not recognize free hexanal, native proteins, free amino acids, other hexanal-amino acid conjugates or other aldehyde, alcohol or ketone protein conjugates. Hexanal concentration of conjugates measured by GC and IC-ELISA were highly correlated ($r = 0.97$), indicating potential use of IC-ELISA as a rapid method to monitor lipid oxidation.

To my husband Matt.
Thank you for all your love and support.

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Chapter 1

INTRODUCTION

Coronary heart disease, strokes and cancer have all been found to be related to dietary fat intake. Atherosclerosis, a major cause of coronary heart disease and a contributor to strokes, has been linked to high fat intake, especially of saturated fats, (Pearson et al., 1983). It has been suggested that polyunsaturated fatty acids, which are more susceptible to autoxidation, may increase the risk of cancer. Studies have shown that restricting calories, polyunsaturated fatty acids and protein can decrease tumor incidence (Pearson et al., 1983). Lipid oxidation has recently received interest as to the possible role it plays in human disease and toxicology (Gutteridge and Halliwell, 1990). Increased levels of malonaldehyde are often associated with certain diseases such as atherosclerosis, diabetes and myocardial infarction (Esterbauer, 1993). Malonaldehyde may also play a role in carcinogenisis by acting as an initiator or promoter (Esterbauer, 1993). Oxidized cholesterol is believed to be a strong atherogenic agent, causing atherogenesis (Kubow, 1993).

Lipid oxidation has also long been recognized as a problem in the food industry. Oxidative rancidity as a result of lipid oxidation is a problem during both the preparation and the storage of food. Both meat and poultry are products susceptible to lipid oxidation. Flavor, color and nutritional changes in raw and cooked meat can occur under any kind of storage conditions (Love and Pearson, 1971).

Lipid oxidation begins with a hydrogen atom being abstracted from the methylene carbon of a fatty acid side chain. Removing the hydrogen atom leaves behind an unpaired electron on the carbon atom, which generally reacts with oxygen to give a peroxyl radical. This radical can combine with other radicals, resulting in termination, or can continue the chain reaction by attacking membrane proteins or removing hydrogens from other fatty acids (Gutteridge and Halliwell, 1990). The primary products of this reaction are hydroperoxides. Their breakdown leads to a mixture of low molecular weight compounds such as alkanes, alkenes, aldehydes, ketones and alcohols (Gray and Monahan, 1992).

This mixture of low molecular weight compounds causes the off flavors associated with rancid or oxidized meat (Gray and Monahan, 1992). One commonly used term for describing oxidized flavors in cooked meats is warmed-over-flavor (WOF). Warmed-over-flavor is described as cardboardy, rancid, stale and metallic and is a problem in both meat and poultry (St. Angelo et al., 1987). Color deterioration also occurs as a result of oxidative reactions. Consumers view the brown color arising from oxidation of ferric hemes as undesirable (Love and Pearson, 1971). Health implications of lipid oxidation have also started receiving attention. Protein and membrane damage may be caused by lipid hydroperoxides and their decomposition products, some of which are believed to be chemical toxicants (Ladikos and Lougovois, 1990). Free radicals that are formed during lipid peroxidation may also have an adverse effect by attacking membrane proteins, leading to impairment of membrane functions (Gutteridge and Halliwell, 1990).

The most commonly used test for measuring lipid oxidation is the 2-thiobarbituric acid (TBA) test. This method was originally believed to only measure malonaldehyde

found in oxidized food (Botsoglou et al., 1994). Since a number of other compounds, such as sucrose, woodsmoke and acetaldehyde, have also been found to react with the acid, the test is now referred to as the thiobarbituric acid reactive substances test, known as TBARS. Because TBARS lacks sensitivity and specificity to MA, it has been widely criticized. Gray and Monahan (1992) suggest using the TBARS test, but to use a second, complementary method (such as hexanal concentration) and to correlate both tests back to sensory scores.

Hexanal is one of the major secondary products formed during lipid oxidation of linoleic acid (Gray and Monahan, 1992). A number of researchers have been studying hexanal as a possible indicator of lipid oxidation. The concentration of hexanal has been found to correlate significantly with sensory scores and TBARS for cooked pork (Shahidi et al., 1987), cooked beef (St. Angelo et al., 1987) and in restructured chicken nuggets (Lai et al., 1995). All of these studies used gas chromatography (GC) to measure hexanal concentration. The drawbacks to this method were outlined by Ajuyah et al. (1993). These include the necessity of dedicated equipment, inconvenient sample analysis (only one sample at a time can be analyzed) and the possibility of peroxide decomposition from the heat used to drive the volatiles onto the GC column.

Enzyme-linked immunosorbent assays (ELISAs) have been developed to test for a variety of substances in food such as bacteria, mycotoxins, pesticides, anabolic agents and adulterants (Samarajeewa et al., 1991). The advantages of ELISAs over GC include reduction in assay time, sample size, cleaning steps and equipment expense. (Samarajeewa et al., 1991). An ELISA for hexanal would provide a reproducible, fast and simple test for monitoring lipid oxidation in meat products.

The overall objective of this project was to develop a monoclonal antibody based ELISA to quantify hexanal in meat to monitor lipid oxidation. The specific objectives of this project were as follows:

a. To produce a monoclonal antibody to hexanal.

b. To develop an indirect competitive ELISA, using that monoclonal antibody, and to test the specificity and sensitivity of the indirect ELISA.

c. To develop a reproducible extraction procedure to extract hexanal from raw and cooked meat and quantify, by ELISA, the amount of hexanal extracted.

d. To correlate the concentration of hexanal, as measured by ELISA, to current accepted protocols, such as GC, in storage studies using meat.

Chapter 2

LITERATURE REVIEW

2.1. Mechanism of Lipid Oxidation

The proposed mechanism of lipid oxidation consists of three phases: initiation, propagation and termination. Initiation involves the removal of a hydrogen atom from the methylene carbon in the side chain of a fatty acid. This results in a radical which preferentially (in an aerobic system) reacts with oxygen to give a peroxyl radical. These radicals can react with each other (termination) or remove hydrogens from other fatty acid side chains, thereby propagating the reaction (Gutteridge and Halliwell, 1990). The reaction was summarized by Asghar et al. (1988) as such :

Initiation:	$LH + O_2$	$\rightarrow$	$L\bullet + \bullet OOH$
Propagation:	$L\bullet + O_2$	$\rightarrow$	$LOO\bullet$
	$LH + LOO\bullet$	$\rightarrow$	$LOOH + L\bullet$
	$LOOH$	$\rightarrow$	$LO\bullet + \bullet OH$
Termination:	$L\bullet + L\bullet$	$\rightarrow$	L-L
	$L\bullet + LOO\bullet$	$\rightarrow$	LOOL
	$LOO\bullet + LOO\bullet$	$\rightarrow$	$LOOL + O_2$

with LH representing the unsaturated fatty acid, LOO• representing the peroxy radical, LO• representing the alkoxy radical and LOOH representing the hydroperoxide.

Unsaturated fatty acids are more susceptible to oxidation than saturated fatty acids due to the allylic CH bonds (Kaur and Perkins, 1991). The bond dissociation energy of the allylic CH structure is 20% less than the bond dissociation energy of a similiar compound that lacks allylic bonds. As a result, the peroxyl radical can readily abstract the hydrogen from the central methylene carbon in a conjugated diene unit (-CH=CH-CH_2-CH=CH), but can not easily remove a hydrogen from a saturated hydrocarbon (Kaur and Perkins, 1991).

2.1.1. Initiation of Lipid Oxidation

The initiation of lipid oxidation begins with the formation of radicals. This can occur through homolysis of weak bonds, such as the O-O bond in a peroxide (Kaur and Perkins, 1991). The classic example is the reduction of hydrogen peroxide by Fe(II), known as the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow \quad Fe^{3+} + HO\bullet + HO^-$$

The resulting hydroxyl radical is extremely reactive and is capable of removing a hydrogen atom from an unsaturated lipid. This reaction is an example of an induced peroxide decomposition.

The source of these metal catalysts is controversial. Heme proteins were found to have a greater effect on lipid oxidation (measured by TBARS) in a pork model system than inorganic iron (Monahan et al., 1993). Ahn et al. (1993), however, found that free

ionic iron had more of an effect on the generation of TBARS in raw turkey meat than ferritin and transferrin, but that heme protein had no effect.

Another way for homolysis of weak bonds to occur is by reacting with singlet oxygen that is produced from absorption of ultraviolet light (Frankel, 1991). Ultraviolet light can also transform carbonyl groups into reactive species that can then behave as reactive free radicals (Kaur and Perkins, 1991).

2.2. Current Methods For Monitoring Lipid Oxidation

Hydroperoxides are the primary products of lipid oxidation. They are colorless, tasteless and odorless. It is their breakdown into a mixture of low molecular weight compounds that results in the rancid and off-flavor characteristics that are associated with oxidized meat. These low molecular weight compounds include alkanes, alkenes, ketones, alcohols, esters and acids (Gray and Monahan, 1992). Sensory analysis is often used to evaluate the extent of lipid oxidation in meat. Warmed-over-flavor, or WOF, is a term used to describe the off flavors resulting from lipid oxidation in cooked meat. This term was first introduced by Tims and Watts (1958). Since the consumer uses organoleptic evaluation when judging the quality of foods, sensory analysis can be a valuable tool. However, sensory experiments can be very time consuming and have poor reproducibility due to differences in odor and taste sensitivity (Gray, 1978). Another problem encountered with sensory testing is that the vocabulary used to describe flavor defects can vary greatly (Frankel, 1991). Some of the terms used are musty, rancid, stale, sour, mettalic and bitter (Civille and Dus, 1992). Also, various storage conditions can result in different flavor descriptions. One technique that has been developed to avoid

some of these problems is quantitative descriptive analysis (Stone et al., 1974). However, this method still involves the need for careful subject selection, training and repeated judgments and a lot of statistical analysis. While any method used to measure lipid oxidation will ultimately need to be correlated to a sensory method (Gray, 1978), a less subjective method needs to be used as the primary determinant of lipid oxidation.

2.2.1. TBA Test

The most common chemical method for measuring lipid oxidation in food is the 2-thiobarbituric acid (TBA) test. This method was developed to measure malonaldehyde (MA). Malonaldehyde is formed in lipid materials either as the by-product of enzymatic thromboxane A_2 production from arachidonic acid or as an end product from nonenzymatic oxidative degradation of polyunsaturated fatty acids (Raharjo and Sofos, 1993). One mole of MA reacts with two moles of thiobarbituric acid to form a pink complex which can then be measured spectrophotometrically at 532 nm (Botsoglou et al., 1994). The extent of oxidation is usually expressed as milligrams of malonaldehyde per kilogram of sample.

The TBA test can be performed (a) by directly heating the sample with the TBA reagent, then measuring the soluble pink complex formed (Turner et al., 1954; Sinnhuber and Yu, 1958), (b) by distillation of the sample to remove volatiles and then reacting the distillate with TBA (Tarladgis et al., 1960; Rhee, 1978), (c) using organic solvents to extract the lipid from the sample and reacting the extract with TBA (Pikul et al., 1983, 1989) or (d) using aqueous trichloroacetic acid (Witte et al., 1970; Newburg and Concon, 1980) or using perchloric acid (Salih et al., 1987; Pikul et al., 1989) to extract the MA

and then reacting the MA with TBA. These methods have been found to correlate well with sensory scores of oxidized meats; however, this test is often criticized because TBA has been found to react with substances other than MDA. For that reason, it is now referred to as the thiobarbituric acid-reactive substances (TBARS) test (Gray and Monahan, 1992).

Several variations of the TBA test have been developed to improve sensitivity and specificity. Botsoglou et al. (1994) have optimized the aqueous extraction method by using third-derivative spectrophotometry in place of conventional spectrophotometry. Third derivative spectrophotometry is used when spectral interference obscures the analytical band. This method has allowed them to develop a rapid, aqueous acid extraction procedure which is sensitive and specific. Another variation of the TBA test was developed which allowed for the measurement of saturated aldehydes. A yellow pigment is formed following the reaction of one mole of aldehyde with one mole of TBA. The reaction can then be measured spectrophotometrically at 455nm (Kosugi and Kikugawa, 1986).

2.2.2. Hexanal

Due to the limitations of the TBARS method, alternative ways of monitoring lipid oxidation have been investigated. Hexanal is a six carbon aldehyde that is one of the major secondary products formed by the oxidation of linoleic acid (Gray and Monahan, 1992). Tamura et al. (1991) measured the amount of reactive products formed in a model oxidation system containing Fe^{2+} and H_2O_2 and different unsaturated fatty acids. The

products were derivatized. *N*-methylhydrazine was used for the α,β-unsaturated aldehydes and the β-dicarbonyl compounds and cysteamine for the saturated normal aldehydes, like hexanal. The quantity of the derivative was measured by GC. Hexanal was one of the major products produced from arachidonic acid (43 nmol/mg) and linoleic acid (141 nmol/mg).

Hexanal concentration has been found to correlate with sensory scores and TBARS values in a variety of products undergoing lipid oxidation. The hexanal concentration and TBA numbers of cooked ground pork, stored for 35 days at 4°C was found to have a correlation coefficient of 0.995 and that as TBA and hexanal numbers went up, flavor acceptability decreased (Shahidi et al., 1987). Hexanal and TBA numbers of freshly cooked, stored and reheated beef muscle showed a high degree of correlation to sensory scores (St. Angelo et al., 1987). Sensory scores from that study indicated that hexanal could be used a marker compound for lipid oxidation. The TBA numbers and the areas of three major GC peaks, one of which was determined to be hexanal, of chicken patties showed a significant positive correlation ($r = 0.97$) (Ang and Young, 1989). The patties had been frozen for about 6 months at -34°C, then thawed, cooked and stored at 4°C for 5 days. A positive correlation was also found between the hexanal concentration, TBA values and sensory scores of restructured chicken nuggets stored at -20°C for 6 months (Lai et al., 1995). These studies demonstrate that hexanal has the potential to be used as an indicator of lipid oxidation in meat.

2.3. Oxidation of Chicken

2.3.1. Fatty Acid Composition of Chicken

More than half of the fatty acids (68.3%) found in chicken fat are unsaturated (Table 2.1.) Since unsaturated fatty acids are highly susceptible to oxidation, it can be expected that chicken meat will oxidize easily and rapidly. The major unsaturated fatty acids in chicken are oleic and linoleic. Formaldehyde is the prominent oxidation breakdown product from oleic acid (Tamura et al., 1991). Hexanal is the major secondary oxidation product of linoleic acid (141 nmol/mg of free fatty acid), followed by malonaldehyde as the next highest product (67.3 nmol/mg of free fatty acid) (Tamura et al., 1991). Because chicken consists of a high concentration of linoleic acid (19.6 % of total fat), it is a good source for hexanal and malonaldehyde during oxidation. As a result, the two methods most commonly used to monitor lipid oxidation in chicken are TBA test and hexanal concentration.

2.3.2. TBA Values for Oxidized Chicken

Rhee et al. (1996) examined the lipid oxidation potential of beef, chicken and pork. They found chicken thigh to have the highest fat content (5.99% raw and 7.46% cooked) and chicken breast the lowest (1.41% raw and 1.92% cooked) of all the meat cuts examined. The TBA value for cooked chicken thigh was 14.5 mg malonaldehyde/kg meat while the TBA value for cooked chicken breast was 7.5 mg malonaldehyde/kg meat, after 6 days storage at 4°C. It has also been determined that the diet of the animal can have an effect on the oxidation potential of meat. The composition of lipids in the subcellular

Table 2.1. Unsaturated fatty acid content of chicken fat.

Unsaturated Fatty Acid		
Abbreviation	Common Name	Content (% of total fat)[1]
14:1	Myristoleic acid	0.3
16:1	Palmitoleic acid	5.7
18:1	Oleic acid	37.3
18:2	Linoleic acid	19.6
18:3	α-Linolenic acid	1.0
20:1	Eicosenoic acid	1.1
20:4	Arachidonic acid	0.1

[1]Adapted from USDA (1979).

membranes is influenced by the fat intake of the diet and these changes reflect a change in susceptibility to peroxidation (Asghar et. al., 1988).

Whang and Peng (1987) measured lipid oxidation in raw chicken skin and muscle. The TBA numbers for chicken thigh increased by a factor of 10 while the TBA numbers for skin and breast doubled after 8 days storage at 4°C. Chicken thigh had a higher concentration of unsaturated fatty acids and oxidized faster than the breast meat.

Freezing and cooking can also have an effect on the rate of lipid oxidation. Pikul et al. (1984) examined the effect of frozen storage and cooking on lipid oxidation in chicken meat. They found a 2.5 fold increase in TBA numbers for both chicken breast and thigh after 6 months storage at -20°C. While freezing decreased the rate of oxidation, oxidation did still occur. The study also showed cooking had an effect on lipid oxidation. The TBA values for the cooked meat, stored at -20°C for 6 months prior to cooking, was 83% higher in malonaldehyde concentration compared to the uncooked sample that had also been stored for 6 months. Freezing rates had no effect on the amount of oxidation in chicken breasts (Tomas and Anon, 1990).

Akamittath et al. (1990) examined the effect of salt, as well as combinations of salt with phosphates and antioxidants. They found that salt had a significant effect on the oxidation of restructured turkey steaks. The TBA number of turkey steaks without salt increased from 0.49 mg malonaldehyde/kg meat to 0.90 mg malonaldehyde/kg meat over 6 days storage at -10°C. The TBA numbers for steaks with salt increased from 1.34 mg malonaldehyde/kg to 3.23 mg malonaldehyde/kg during the same storage period.

Phosphates have some antioxidative effect in turkey steaks, but the prooxidant effect of salt and other free ions was eventually able to overcome the antioxidant effect.

2.3.2. Hexanal Concentration in Oxidized Chicken

Although a large number of researchers have used hexanal as a marker compound for oxidation in chicken, most have used GC to quantify hexanal. The values obtained have varied widely, mainly due to differences in cooking, storage conditions and additives, all of which affect the extent of lipid oxidation, as discussed above. The GC methods most commonly used for monitoring flavors in foods were outlined by Reineccius (1996). The most commonly used method to isolate flavor compounds prior to GC anaysis is headspace concentration, in which the sample is purged with an inert gas and the volatiles trapped. The volatiles are then removed from the trap either thermally, by heating, or chemically, using a solvent.

Ramarathnam et al. (1991) used headspace concentration followed by solvent extraction of the volatiles using *n*-pentane. They determined the hexanal concentration, after 24 hour storage at 4° C, to be 9.84 μg/g meat for uncured chicken (ground chicken cooked in a water bath with no additives) and 0.11 mg/kg for cured chicken (salt, sugar, sodium ascorbate, sodium tripolyphosphate and sodium nitrate were added to the chicken prior to cooking). The hexanal concentration of cooked white and dark meat of chicken was found to have increased from 64 ng/g meat at day zero to 314 ng/g meat after 15 days at 4°C for the white meat and from 15 ng/g meat to 1395 ng/g meat after 10 days for the dark meat (Ajuyah et al., 1993). This study used a modified headspace technique. During

the purging step, the volatiles were trapped in a flask submerged in liquid nitrogen. The resulting distillate was then allowed to thaw at room temperature prior to injection onto the GC. Another study looked at the concentration of hexanal in roast chicken after 5 days storage at 4°C (Dupuy et al., 1987). The concentration was measured using a combination of headspace concentration and direct injection. The concentrations were measured on days 0, 1, 3 and 5 and were found to be 0.09, 6.9, 10.8 and 14.6 μg/g meat respectively. The hexanal concentration of chicken nuggets, which had been fried, then frozen for 6 months without any antioxidants, was found to be 18.5 ug/g meat (Lai et al., 1995). Hexanal concentration was determined using headspace concentration with thermal desorption of the volatiles from the traps.

Although hexanal concentration by GC has been used by a number of researchers, analysis by GC can be a long and involved process. Some of the drawbacks of GC include expensive equipment, being able to analyze only one sample at a time and the possibility of peroxide decomposition to additional volatiles by the heat used to drive the extracted volatiles onto the GC (Ajuyah et al., 1993). An alternative to GC would be an enzyme linked immunosorbent assay (ELISA) to measure hexanal concentration. These assays have the advantages of reduced assay time, sample size and equipment expense compared to GC (Samarajeewa et al., 1991). An ELISA for hexanal would provide a faster and simpler way to monitor lipid oxidation.

2.4. ELISA Development

Antibodies are proteins produced by the body in response to foreign molecules. An antibody binds specifically to an antigen and this characteristic is the reason a variety of methods have been developed utilizing this interaction (Harlow and Lane, 1988). One kind of immunoassay that is commonly used is ELISA. ELISAs involve the use of enzyme labeled antibodies or antigens and take advantage of the antibody-antigen complex that is formed. The first part of developing an ELISA is producing an antibody.

2.4.1. Conjugate Production and Determination of Modification

In order for a compound to elicit an immune response, which is the first step in antibody production, it must be immunogenic. Size is generally the determining factor of immunogenicity. Compounds with a molecular mass of less than 5000 daltons generally are too small to elicit an immune response and are referred to as haptens (Harlow and Lane, 1988). Haptens can be made immunogenic by conjugating them to proteins. This conjugate is then large enough to cause an immune response. Hexanal, at 100 daltons, is a hapten and would require conjugation to a protein to become immunogenic.

Ketones and aldehydes, like hexanal, can react with primary amines to form imines, which are also known as Schiff bases (Wade, 1987). The reaction is a condensation, involving the joining of two organic molecules with the elimination of water (Figure 2.1). However, the double bond in the Schiff base is unstable. Sodium cyanoborohydride will reduce this double bond to a single bond (Jentoft and Dearborn, 1979). This results in a stable conjugate. Means and Feeney (1968) determined that it is

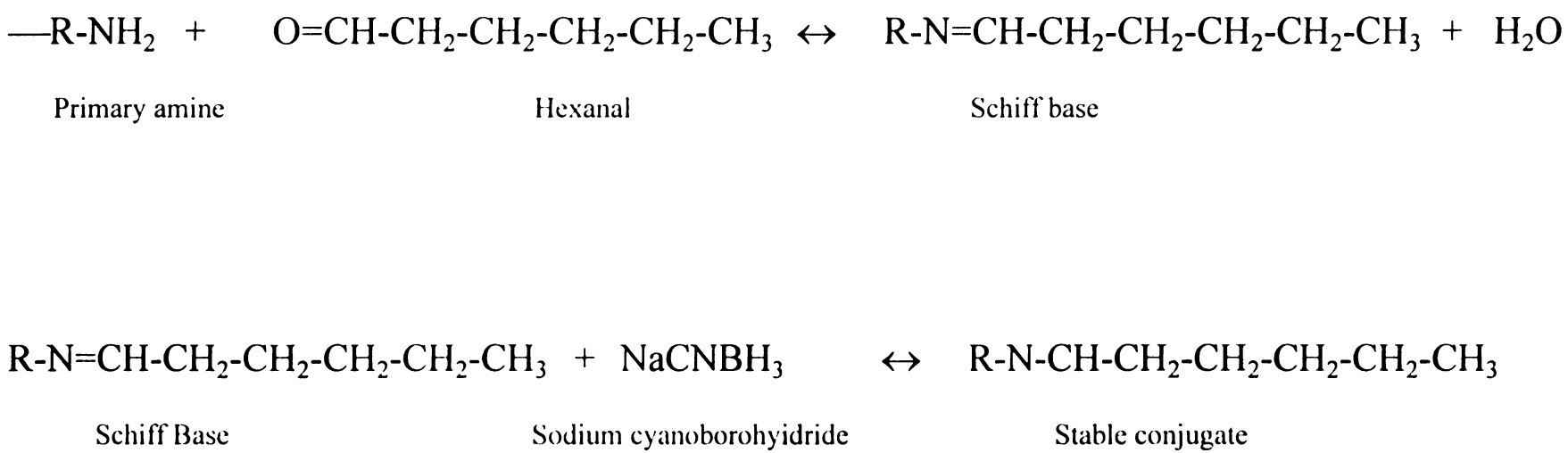

Figure 2.1. Condensation reaction of hexanal and a primary amine that results in a Schiff base. The Schiff base is reduced to a stable conjugate with sodium cyanoborohydride.

only the ε-aminolysine groups of the proteins that are modified when aliphatic aldehydes are added with sodium cyanoborohydride at pH 9 and 0°C.

Hexanal can be conjugated to proteins through the Schiff base reaction. A research group looked at the ability of acetaldehyde, an aldehyde smaller than hexanal, to bind to bovine serum albumin (Donohue et al., 1983), and found both stable and unstable adducts were formed. The unstable adducts were reduced to form stable adducts by using sodium cyanoborohydride. The optimal reaction conditions for reducing the unstable adducts to stable ones were determined to be a pH of 9.5, about 0.1 mM sodium cyanoborohydride and a reaction time greater then 60 min at room temperature.

To use these conjugates for immunizations or as antigens in immunoassays, it is necessary to know how much of the protein has been modified, as an indirect way to determine the concentration of the compound conjugated to the protein. One way to do this is to determine the number of free amino groups that are left on the protein after modification (Habeeb, 1966). This is accomplished by using 2,4,6-trinitrobezenesulfonic acid (TNBS) to react with free amino groups. The absorbance is read at 335 nm and it was found that there is a linear relationship between absorbance and free amino group concentration.

2.4.2. Monoclonal Antibody Production

Once a suitable conjugate has been made, or if the compound of interest is immunogenic without conjugation, monoclonal antibody production can be started. There are three characteristics of monoclonal antibodies that define their usefulness- specificity

for binding with an antigen, homogeneity (they are produced from only one clone and are not a mixture of antibodies) and ability to produce monoclonal antibodies in unlimited quantities (Harlow and Lane, 1988). Monoclonal antibodies were first reported by Kohler and Milstein (1975). Monoclonal antibodies are produced by hybridoma cells, which are hybrids between myeloma cells and spleen cells. The spleen cells are obtained by removing the spleen from mice or rats which have been immunized with the antigen and homogenizing the tissue. Generally, the easiest to use are BALB/c mice or LOU rats because most of the myelomas commonly used for fusions are derivatives of mylomas from these species (Galfre and Milstein, 1981). The first step in the process of producing a monoclonal antibody is to begin immunizing mice or rats. The injection is prepared by emulsifying 1-5 mg/mL of the antigen with an equal volume of Freund's complete adjuvant (Galfre and Milstein, 1981). Injections are then repeated at 3-5 week intervals, using an emulsion of the antigen with Freund's incomplete adjuvant. About 10 days after injecting, a sample of blood is taken and tested for antibodies using an immunoassay. The animals producing antibodies with high specificity and avidity are used in the fusion. The animals are sacrificed and their spleens removed and homogenized. Spleen cells are not able to survive in tissue culture, but by fusing them with myeloma cells, which can survive in tissue culture, a hybrid that can survive in culture indefinitely is obtained. Polyethylene glycol (PEG) is used to dissolve the cell walls and allow the fusion to occur. The fused cells are selected using medium containing hypoxanthine, aminopterin and thymidine (HAT medium). The myelomas cells are mutants that lack either the enzyme hypoxanthine guanine ribosyltransferase or thymidine kinase. These mutants cannot grow in a medium containing aminopterin supplemented with hypoxanthine and thymidine.

Since unfused spleen cells can not survive in culture, it is only the fused cells that are capable of growing. After fusion, individual hybridomas producing the desired antibodies can be selected and further cloned.

2.4.3. Classification of ELISA Types

Once an antibody has been produced, an ELISA format needs to be selected. ELISAs are generally classed as one of two types. They are either competitive or non-competitive (Clark and Engvall, 1980). Competitive ELISAs are ones where the unlabeled antigen and the enzyme labeled antigen compete for a limited number of antibody sites. Non competitive ELISAs are where the antigen or antibody that is to be measured are allowed to react with an excess of the other reactant.

The first kind of competitive ELISA is when the antibody is attached to a solid support. Any unattached antibody is washed away and a fixed concentration of enzyme labeled antigen is added with either a known concentration of standard antigen or an unknown concentration of test antigen. After washing again to remove the unreacted antigen, the enzyme labeled antibody-antigen complex is incubated with a substrate. The enzyme reaction is stopped and the concentration determined spectrophotometrically. The measured end product is inversely proportional to the concentrations of standard antigen or test antigen.

The second kind of competitive ELISA involves attaching the antigen to the support and using an enzyme labeled antibody (Figure 2.2.). The free antigen that is

1. Attachment of the antigen.

2. Incubate with standard or sample and antibody.

3. Incubate with enzyme labeled second antibody followed by substrate and measure the end point.

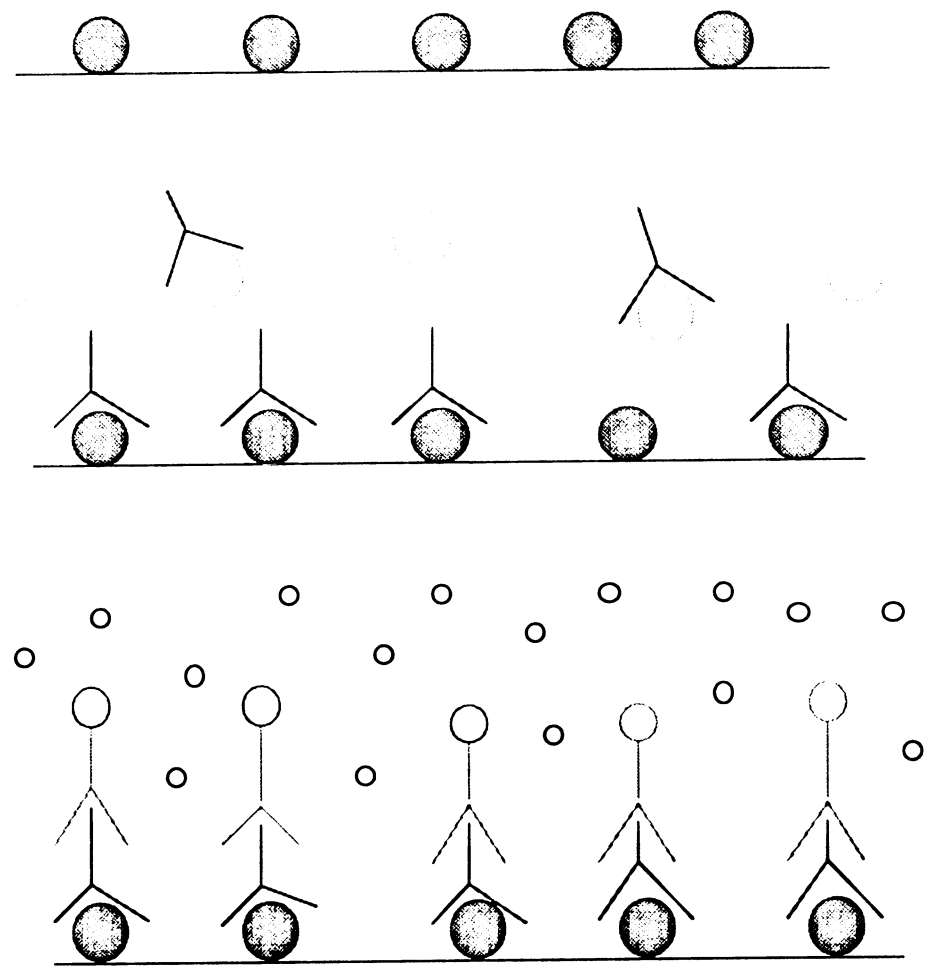

Figure 2.2. Diagram of indirect competitive ELISA.

added as a standard with the labeled antibody competes for the antibody with the attached antigen. A variation of this is when an enzyme labeled second antibody is used, one that is specific to the IgG of the animal species the first antibody was produced in. In this case, unlabeled antibody is added with the free antigen. After washing, the unlabeled antigen-antibody complex is incubated with enzyme-labeled anti-IgG. A substrate is added and the concentration is quantitated as above (Clark and Engvall, 1980).

There are two main kinds of non-competitive ELISAs. The first type is a single site or one antibody assay. The antibody is attached and standard or test antigen is allowed to react with the antibody. After washing, excess enzyme labeled antigen is added and will attach to any unreacted antibody. Substrate is added after washing and the reaction stopped and measured as in the competitive ELISA. The concentration of the standard or the sample in this ELISA is inversely proportional to the measured end product. Another kind of single site non-competitive ELISA is when test or standard antigen is allowed to react with an excess of enzyme labeled antibody. The reaction mixture is then added to an excess of immobilized antigen. As above, the concentration is inversely proportional to end product.

The second kind of non-competitive ELISA is a two site or sandwich ELISA involving two antibodies. Attached antibody is incubated with the test or standard antigen. After washing, an enzyme labeled second antibody is added. As a result, the concentration is proportional to the end product (Clark and Engvall, 1980).

The ELISA format chosen depends on the type of antibody available and the purity of the antibody, as well as whether it is the presence or quantity of antigen or

antibody that is to be measured (Harlow and Lane, 1988). Competitive ELISAs are highly specific, however, their use is limited when the test solution contains serum, urine or tissue extracts (Clark and Engvall, 1980). These solutions may contain enzymes such as proteases and noncompetitive enzyme inhibitors that may alter the activity of the enzyme labeled antigen or antibody if they are incubated with it. This problem is avoided by using a noncompetitive ELISA.

2.5. Examples of ELISAs Currently in Use

2.5.1. ELISAs in the Food Industry

ELISAs have been used to detect a variety of compounds in food (Samarajeewa et al., 1991) such as bacteria, molds, viruses, pesticides, anabolic agents, and adulterants. In the meat industry, ELISAs have been used for detection of the meat species or minor meat protein, detection of non-meat products in meat, as well as testing for *Salmonella* and *Staphylococcal* enterotoxins in meat products (Fukal, 1991). Concern about proper cooking has led to the development of ELISAs to enzymes that denature as they are heated. Monoclonal antibodies were used in a sandwich ELISA to monitor endpoint cooking of poultry breasts by testing for lactate dehydrogenase, a protein that denatures during cooking and can therefore be used as an indicator of the final temperature (Abouzied et al., 1993).

Other food contaminants, such as pesticides and mycotoxins, can also be tested for by ELISA. Mycotoxins are secondary metabolites produced by molds. They are of low molecular weight and must be conjugated to a carrier protein before they are capable

of producing an immune response (Pestka et al., 1995). Even though the compounds are small, a large number of ELISA kits are commercially available for detecting a wide range of mycotoxins, such as aflatoxin, ochratoxin, deoxynivalenol, and zearalenone.

2.5.2. Acetaldehyde ELISAs

Outside of the food industry, ELISAs are being used for detection of acetaldehyde adducts that are formed when acetaldehyde condenses with plasma proteins (Israel et al., 1992). Acetaldehyde is the primary product of ethanol metabolism and is believed to cause hepatic injury by binding to macromolecules (Donohue et al., 1983). This research group examined the ability of acetaldehyde to bind to proteins, specifically, bovine serum albumin (BSA), and found that both stable (reduced bonds) and unstable (unreduced bonds) adducts were formed between acetaldehyde and BSA.

Monoclonal and polyclonal antibodies have been produced to acetaldehyde conjugated to human plasma proteins (Israel et al., 1986). This group determined that both monoclonal and polyclonal antibodies specifically recognized the conjugated proteins, with no recognition of unmodified erythrocyte proteins. They also found that the adducts were formed with the lysine side chains of the proteins and that the polyclonal antibodies were specific to the lysine-aldehyde adduct, with no recognition of adducts formed with tyrosine or valine.

Another monoclonal antibody to acetaldehyde adducts was produced by a different research group (Klassen et al., 1994). This group determined whether the antibody could differentiate between an adduct formed under reducing conditions as opposed to non-reducing conditions. Sodium cyanoborohydride was used as the reducing

agent. They found one clone that was able to recognize all adducts formed under reducing conditions, regardless of the carrier protein. This same antibody was then further tested to determine its specificity (Thiele et al., 1994). The antibody was found to be specific to acetaldehyde adducts that were formed with *N*-ethyl lysine under reducing conditions. The antibody did not recognize adducts formed with arginine, ethylamine or lysine under reducing conditions or with proteins modified by acetaldehyde under non-reducing conditions. The theory that the *N*-ethyl lysine adduct is the epitope was further tested using mouse and human growth factor (EGF). Both contain one alpha amino group, but only the human-EGF has lysine residues with epsilon amino groups. The antibody reacted with the human-EGF only.

2.5.3. Polyclonal Antibody ELISA for Hexanal

A polyclonal antibody and ELISA for the detection of hexanal-protein conjugates was developed by Smith (1997). The antibody was characterized and found to cross-react with heptanal (86.3%) and pentanal (11.8%). These two compounds are the aliphatic aldehydes with one carbon more and less than hexanal. The limit of detection of the polyclonal antibody ELISA was determined to be 7.4 ng hexanal/mL. This ELISA was then used to monitor lipid oxidation in a model meat system and the results were compared to those from a TBA assay and a GC method for hexanal. The ELISA was found to correlate well with both the TBA ($r = 0.85$) and the GC ($r = 0.89$) methods.

2.6. Conclusion

One of the major secondary products of linoleic and arachidonic acid oxidation, hexanal, has been shown to be a suitable marker of lipid oxidation in different meats. An ELISA to hexanal would allow for rapid detection of hexanal and would be a useful alternative to GC. Polyclonal antibodies have already been produced to hexanal, but cross-react with two other aliphatic aldehydes. Monoclonal antibodies for hexanal could prove to be more specific to hexanal, as well as more sensitive. Monoclonal antibodies that are both specific and sensitive have been produced to acetaldehyde, an aldehyde that is smaller than hexanal. The development of a competitive, monoclonal antibody ELISA to monitor lipid oxidation is feasible and could prove to be an invaluable asset to producers and researchers.

Chapter 3

PRODUCTION AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY AGAINST A HEXANAL-PROTEIN CONJUGATE

3.1. ABSTRACT

Monoclonal antibodies were produced to hexanal conjugated to bovine serum albumin (BSA). The final clone was designated 45P1-D9 and determined to be an immunoglobulin G_1 isotype. An indirect competitive ELISA was developed and used to determine the epitope recognized by the antibody. The working range of the ELISA was 1 to 50 ng hexanal/mL. Hexanal conjugated to three different proteins [BSA, chicken serum albumin (CSA) and keyhole limpet hemocyanin (KLH)] was recognized, while free hexanal and the native proteins were not. The antibody cross-reacted with pentanal, heptanal and 2-*t*-hexenal conjugated to CSA with cross-reactivities of 37.9%, 76.6% and 45.0%, respectively. There was less than 10% binding inhibition (at 100 ng/mL of aldehyde) with propanal, butanal, octanal and nonanal conjugated to CSA. Conjugates made from binding the branched aldehydes, alcohols and a ketone to CSA also gave less than 10% inhibition of binding at the same concentration. The antibody showed no recognition of the glycine or arginine conjugates or the unmodified amino acids (less than 3% inhibition of binding at 2 mM). The antibody showed recognition of the lysine conjugate (17% inhibition at 2 mM), but showed higher recognition of the ε-amino

caproic acid conjugate (47% inhibition at 2 mM). ε-Amino caproic acid is a lysine derivative without the α amine group, simulating a lysine contained in a protein. The results indicate that the antibody recognizes the lysine-hexanal complex and is specific to this complex to within plus or minus one carbon.

3.2. INTRODUCTION

Lipid oxidation can result in objectionable flavors which are generally the deciding factor in the storage life of foods (Frankel, 1993). Lipid oxidation can also cause changes in color and nutritional value under a range of storage conditions (Love and Pearson, 1971). These changes are usually viewed by consumers as undesirable. The safety of oxidized products has also been questioned. Malonaldehyde (Esterbauer, 1993) and oxidized cholesterol (Kubow,1993) are both being investigated for their possible roles in disease. The radicals formed during oxidation are capable of attacking membranes, leading to impairment of membrane function (Gutteridge and Halliwell, 1990). Both the safety concerns and the economic losses, resulting from consumer rejection of products, show there is a need for a fast and reliable indicator of oxidation in food.

Lipid oxidation begins with the removal of a hydrogen atom from the side chain of a fatty acid. This results in a radical which can combine with other radicals (termination) or can continue to react by removing hydrogens from other fatty acid side chains (Gutteridge and Halliwell, 1990). Hydroperoxides are the primary products of lipid oxidation and are tasteless, colorless and odorless. The breakdown of

hydroperoxides produce the low molecular weight compounds which give oxidized food its characteristic odor and flavor (Gray and Monahan, 1992). One of the major secondary low weight molecules that is formed from the oxidation of linoleic acid is hexanal (Gray and Monahan, 1992). Monitoring hexanal concentration was recommended as a possible alternative to the thiobarbituric acid (TBA) test (Gray and Monahan, 1992) and has been used successfully by a number of researchers for assessing the extent of lipid oxidation in foods.

Hexanal concentration has been correlated to sensory scores for cooked pork (Shahidi et al., 1987), cooked beef (St. Angelo et al., 1987), restructured chicken nuggets (Lai et al., 1995), oat, corn and wheat cereals (Fritsch and Gale, 1977), peanuts (Bett and Boylston, 1992) and vegetable oil (Warner et al., 1978). These studies used gas chromatography (GC) to measure the concentration of hexanal. Gas chromatographic methods have a number of drawbacks including the need for dedicated equipment, inconvenience of large sample analysis (only one sample can be analyzed during each run) and possible decomposition of peroxides to additional volatiles from the heat used in the analysis of the sample (Ajuyah et al., 1993).

ELISAs have been developed to detect or quantify a variety of substances in food including pesticides, adulterants and mycotoxins (Samarajeewa et al., 1991) and are accepted as alternatives to GC. The advantages of ELISAs over GC include reduction in assay time, sample size, cleaning steps and equipment expense (Samarajeewa et al., 1991). An ELISA to quantify hexanal would provide a fast and simple test for monitoring lipid oxidation in foods.

Polyclonal antibodies have been produced to hexanal-protein conjugates prepared by conjugating hexanal to protein via Schiff base reactions with the lysine side chains (Smith, 1997). These antibodies were characterized using an indirect competitive ELISA. The antibodies recognized hexanal-protein conjugates but did not recognize free hexanal or the native protein. The immunodominant epitope was proposed to be the hexanal modified lysine. The antibody was also found to cross-react with the pentanal, 2-*t*-hexenal, (heptanal) and (octanal) (5 to 8 carbon aliphatic aldehydes), as well as 2-methylbutanal, a branched aldehyde. The amount of cross-reactivity was 11.8%, 100%, 86.3%, 2.2% and 1.1%, respectively. The results indicated the polyclonal antibody was most specific to the aliphatic aldehydes within one carbon length of hexanal but did not recognize the branched aldehydes with similar carbon numbers, indicating that these antibodies were fairly specific. The limit of detection for the polyclonal antibody was 7.4 ng hexanal/mL.

A monoclonal antibody might be more sensitive and specific for hexanal than the polyclonal antibody. Monoclonal antibodies have been produced to acetaldehyde, a two carbon aldehyde (Klassen et al., 1994). One of the clones recognized reduced acetaldehyde-protein conjugates, regardless of the carrier protein, but did not recognize unreduced conjugates or unmodified proteins when used in an indirect ELISA. This antibody was found to have a limit of detection of 10 ng/mL. This clone was further analyzed and it was found that the antibody recognized only the *N*-ethyl lysine adducts that were formed when the protein was modified by acetaldehyde (Thiele et al., 1994). These studies demonstrate that it is possible to produce a monoclonal antibody specific to a low carbon number aldehyde.

The purpose of this study was to produce monoclonal antibodies to hexanal and then to develop and optimize an ELISA using antibodies from the clone that showed the highest sensitivity and specificity. Following optimization, the epitope the antibody recognized was characterized to determine if the immunoassay might be used to monitor lipid oxidation in actual food systems.

3.3. METHODS AND MATERIALS

3.3.1. Materials

Female mice (BALB/c, 6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Freund's complete and incomplete adjuvants were purchased from Difco Laboratories (Detroit, MI). The myeloma cell line P3/NS 1/1-Ag4 (NS-1) (ATCC TIB 18) was obtained from American Type Culture Collection (Rockville, MD). Tissue culture plastic ware was purchased from Corning Laboratory Science Co. (Corning, NY). Microtiter wells (Immunolon-2 Removawells) were from Dynatech Laboratories (Alexandria, VA). Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (GAM-IgG HRP) was purchased from Cappel Laboratories (West Chester, PA). The TMB (3,3',5,5' tetramethyl benzidine) substrate and stopping buffer were purchased from Di-Agra (Sterling Heights, MI). Propanal, butanal, pentanal, heptanal, nonanal, 2-methylpentanal, 2-methylbutanal, 3-methylpentanal, 2-hexanol, hexanol and 2-heptanone were all purchased from Aldrich Chemical Co. (Milwaukee, WI). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

3.3.2. Conjugate Preparation

The conjugates were prepared as described by Smith (1997), except that the final sodium cyanoborohydride concentration was reduced to 20 mM and as a result, no dialysis was needed. The proteins used in this study were bovine serum albumin (BSA), chicken serum albumin (CSA) and keyhole limpet hemocyanin (KLH). The hexanal modified proteins were produced by adding 246 μL of hexanal to 9 mL of 16.7 mg/mL protein dissolved in phosphate buffered saline (PBS, pH 7.4; 0.1 M sodium chloride, 0.01 M sodium phosphate, pH 7.4). After vortexing, 1 mL of 200 mM sodium cyanoborohydride ($NaCNBH_3$) dissolved in 0.1 N NaOH was added to the hexanal-protein solution to reduce Schiff bases. The final concentrations of this reaction mixture were: 15 mg/mL protein, 200 mM hexanal and 20 mM $NaCNBH_3$. The unreduced conjugates were prepared the same way except that no $NaCNBH_3$ was added. The reaction was allowed to proceed overnight at room temperature. Modification was determined by measuring the loss of reactive amino groups in the conjugates, as compared to the corresponding native protein, by the trinitrobenzenesulfonic (TNBS) acid test of Habeeb (1966) as modified by Smith (1997). Conjugates were diluted to 1 mg/mL with PBS (pH 7.2; 0.1 M sodium chloride, 0.01 M sodium phosphate), aliquotted and frozen at -20°C.

Amino acid analysis was performed by Dr. Nathalie Trottier's lab (Department of Animal Science) using high performance liquid chromatography. The amino acid content of hexanal modified CSA, BSA and KLH, as well as the native form of these proteins, was determined following derivatization of the sample with phenylisothiocyanate (Cohen

et al., 1989). Briefly, a volume of each conjugate or native protein, equaling 20 mg of protein, was hydrolyzed with 2.5 mL of 4 mM norleucine and 25 mL of 6N HCl by autoclaving the sample mixtures for 24 h. The samples were then brought up to a volume of 40 mL using HPLC grade water. For the conjugation, phenylisothiocyanate was added to 25μL of the hydrolyzed sample. The samples were then analyzed by HPLC to determine the amino acid content. The limit of detection for this method was 1 picomole. Results were expressed as molar percentage for each amino acid. Experiments were run in duplicate.

3.3.3. Production of Monoclonal Antibodies

Two groups of 6-8 week old Balb/c mice were injected subcutaneously with 50 μg of hexanal modified protein conjugates (conjugates were produced and checked for modification as described in above section) in 0.1 mL 0.8% saline and emulsified with 0.1 mL of Freund's complete adjuvant. Mice were injected with a total volume of 0.5 mL. Five mice were injected with the BSA-hexanal conjugate and five with the KLH-hexanal conjugate. Booster injections were given at 2 week intervals with the same injection except that Freund's incomplete adjuvant was used instead of the complete adjuvant. Two weeks after the second booster injection, the mice were bled (from the tail), titers were determined and the sera tested for antibody by an indirect competitive ELISA. The two mice whose serum showed the lowest recognition of the native BSA, when tested by an indirect competitive ELISA, were selected for the fusion. A final injection, containing 50

μg of hexanal modified protein in 0.1 mL 0.8% saline, was given three days prior to the fusion.

The fusion procedure followed was performed as described in Galfre and Milstein (1981) and as modified by Abouzied et al. (1990). Briefly, the mice were sacrificed and the spleen removed. Mouse spleen cells ($2x10^8$) were mixed with $2x10^7$ NS-1 cells using 50% polyethylene glycol as the fusing reagent. Following fusion, the cells were suspended in Dulbecco's modified medium containing 20% fetal bovine serum (FBS), 1% NCTC medium, 10 mM MEM sodium pyruvate solution and penicillin/streptomycin solution (100 U/mL) and distributed into 96 well culture plates. The plates were incubated at 37°C in a humid atmosphere containing 7% CO_2. After 24 h of incubation, half of the media in the wells was removed and replaced with an equal volume of hypoxanthine, aminopterin and thymidine selective medium (HAT medium). The cells were fed in this manner every three days. After two weeks, the HAT medium was replaced with HT medium (HAT medium without the aminopterin). The wells showing growth and color change were tested for antibody production using an indirect competitive ELISA. Hybridomas that showed production of antibodies to hexanal-protein conjugates were expanded and cloned twice by limiting dilution (Goding, 1980). The hybridomas whose antibodies showed the highest percent inhibition were transferred to larger growth vessels.

Hybridomas were stored in FBS-dimethyl sulfoxide (9:1) under liquid nitrogen (Abouzied et al., 1990). Supernatant from the cell culture of the final clone was collected and the antibody was purified by precipitation with 50% ammonium sulfate (Hebert et al.,

1973) then dialyzed for two days against PBS (pH 7.2) at 4°C. The purified antibody was then aliquotted and frozen at -20°C. The immunoglobulin subclass of the antibody secreted by the final clone was determined by Sigma Immunotype™ Mouse Monoclonal Antibody Isotyping Kit.

3.3.4. Titer Determination and Screening by Indirect ELISA

An indirect ELISA (Abouzied et al., 1990) used for titer determination was performed by coating microtiter plates overnight at 4°C with 100 μL hexanal-KLH or hexanal-BSA conjugate (1μg protein/mL), diluted in 0.1 M carbonate buffer (pH 9.6). Plates were washed 4 times with PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T). The non-specific binding sites were then blocked by adding 300 μL of 0.5% casein in PBS (pH 7.2) to each well and incubating 30 min at 37°C. After washing 4 times with PBS-T, 50 μL of serially diluted sera (in PBS; pH 7.2) were added and incubated for 30 min at 37°C. After incubation, the plates were again washed 4 times with PBS-T. To each well, 100 μL of GAM-IgG HRP, diluted 1:500 in PBS with 0.5% casein, was added. After incubating 30 min at 37°C, the plate was washed 8 times with PBS-T. Peroxidase binding was determined using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-H_2O_2 substrate as described by Pestka et al. (1982). Absorbance was measured at 405 nm using a THERMOmax plate reader (Molecular Devices, Menlo Park, CA). The titer of the serum was arbitrarily determined as the dilution showing an absorbance that was twice the absorbance of the pre-immune serum at the same dilution.

The indirect competitive ELISA used for screening the mice and hybridomas for antibody production was the same as above except that after blocking the nonspecific sites, 50 μL of cell culture supernatant or sera were added with either 50 μL of PBS (pH 7.2) as the zero concentration or with 50 μL hexanal modified KLH at 10 μg protein/mL as the competition. The percent inhibition at 10 μg protein/mL was determined by dividing the absorbance at that concentration by the absorbance at zero concentration then subtracting from 1.

3.3.5. Protein Specificity by Indirect Competitive ELISA

Hexanal modified and native CSA, BSA and KLH were tested for antibody recognition. The indirect competitive (IC) ELISA used was performed by coating microtiter plates overnight at 4°C with 100 μL hexanal-CSA conjugate (1μg protein/mL), diluted in 0.1M carbonate buffer (pH 9.6). Plates were washed 4 times with PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T). Purified antibody (50 μL of 1.6 μg protein/mL in PBS, pH 7.2) was added with 50 μL of hexanal modified or native protein at concentrations of 0 to 10 μg protein/mL and incubated for 15 min at room temperature. After incubation, the plates were again washed 4 times with PBS-T. To each well, 100 μL of GAM-IgG HRP, diluted 1:500 in PBS with 0.5% casein, was added. After incubating 15 min at room temperature, the plate was washed 8 times with PBS-T. Peroxidase binding was determined using a TMB buffer. After 10 min, the reaction was stopped with stopping buffer and the absorbance was read at 450 nm. A standard curve was generated by plotting the concentration of hexanal modified protein against binding inhibition.

Since each protein contains different amounts of lysine, the hexanal concentration was calculated from the protein concentration, based on the moles lysine/mole protein and the percent modification. The following formula was used, showing the conversion of protein concentration to hexanal concentration:

$$\frac{\mu g\ protein}{mL} \times \frac{moles\ lysine}{1\ mole\ protein} \times \frac{1\ mole\ protein}{MW\ (g/mole)\ protein} \times \frac{1\ gram}{10^6\ \mu g} \times \frac{10^6\ \mu moles}{1 mole} = \frac{\mu moles\ lysine}{mL}$$

The μmoles of lysine are multiplied by the % modification to adjust for differences in lysine modification during the conjugation. Since one mole of lysine will bind one mole of hexanal, these concentrations are equal :

$$\frac{\mu mole\ hexanal}{mL} \times \frac{100\ g\ (MW\ of\ hexanal)}{1\ mole} \times \frac{1\ mole}{10^6\ \mu mole} \times \frac{10^9\ ng}{1\ g} = \frac{ng\ hexanal}{mL}$$

The moles of lysine in each protein were calculated from the amino acid analysis. The percent mole of lysine was multiplied by the molecular weight of the protein to obtain the molecular weight contributed by lysine. This was divided by the molecular weight of lysine (minus 18, for the loss of a water when it is a part of a protein) to obtain the moles of lysine per mole of protein. The molecular weight used for KLH was 1700 kD, the average range for the commercial protein. The molecular weights used for BSA and CSA were 66,000 and 66,300 daltons, respectively. An example of this formula, converting 1 μg protein/mL concentration of hex-CSA to hexanal concentration is shown in Appendix

A. Standard curves were generated by plotting the concentration of hexanal against the binding inhibition produced by each protein conjugate.

The antibody was also tested for recognition of free hexanal using the above ELISA format. Different aliquots of hexanal were added to 30% methanol so that the final concentration of free hexanal was 0, 0.05, 0.1, 0.25, 0.5 and 1.0 μg/mL. Unreduced conjugates (prepared as stated under the conjugate preparation section) were also tested for recognition in the indirect competitive ELISA at concentrations of 0 to 5 μg protein/mL.

3.3.6. Precision

Intra-assay and inter-assay precision, or reproducibility, of the IC-ELISA was determined (Deshpande, 1996). The concentrations used for determining the precision were 0, 5, 10, 20 and 40 ng/mL of hexanal, which corresponds to 100, 80, 65, 55 and 50% of the maximum binding (B_O), respectively. The average, standard deviation and coefficient of variation was determined for all replicates at each concentration. The intra-assay variability was determined using 16 replicate wells and the inter-assay variability was determined using 32 replicate wells from eight different plates.

3.3.7. Antibody Cross-Reactivity by IC-ELISA

Conjugates were produced, using the conjugation procedure in section 3.3.2., by replacing either the hexanal or the protein portion of the conjugate. CSA was modified with the following compounds in place of hexanal: propanal, butanal, pentanal, heptanal,

octanal, nonanal, 2-methylpentanal, 2-methylbutanal, 3-methylpentanal, 2-*t*-hexenal, 2-hexanol, hexyl alcohol and 2-heptanone. The final concentrations of these reactions were: 15 mg/mL protein, 200 mM hexanal substitute and 20 mM $NaCNBH_4$. The standard curve used in the ELISA was converted from CSA concentration to aldehyde, alcohol or ketone concentration in order to compare the ELISA results to those obtained by GC, which is generally expressed as microgram or milligram of hexanal per gram of sample. The formula for converting protein concentration to hexanal concentration was used, but substituting the molecular weight of each reactant for that of hexanal (100 daltons).

Hexanal was used to modify the following amino acids: glycine, L-arginine, L-lysine and ε-aminocaproic acid. The final concentrations of these reactions were: 200 mM amino acid, 200 mM hexanal and 20 mM $NaCNBH_3$. The amount of modification of all conjugates was determined using the TNBS assay.

The cross-reactivity of the monoclonal antibody was determined by testing the aldehyde, alcohol, ketone and amino acid conjugates in the IC-ELISA described in section 3.3.5. The conjugates were added with the antibody at concentrations of 0 to 100 ng/ml of aldehyde, alcohol or ketone, or 0 to 2 mM amino acid. Cross-reactivity was determined by dividing the concentration of hexanal-CSA required for 50% binding inhibition by the concentration of aldehyde, alcohol, ketone or amino acid conjugate required for 50% binding inhibition multiplied by 100. All determinations were made in at least triplicate.

3.3.8. Statistics

Standard error of the means was determined for each curve, at each concentration, using one way analysis of variance (SAS, SAS Institute, Inc., Cary, NC).

3.4. RESULTS AND DISCUSSION

3.4.1. Antigen Preparation

Amino acid analysis of the native and modified CSA, BSA and KLH showed the lysine content of the hexanal modified proteins to have decreased to an undetectable concentration. The lysine content of native CSA, BSA and KLH was 8.84, 10.79 and 4.37 mole percent, respectively (Table 3.1.). The calculated moles of lysine per mole of protien, based on the amino acid analysis, were 580, 56 and 46 for KLH, BSA and CSA, respectively. Harlow and Lane (1988) and Alaiz and Giron (1994) reported 59 moles of lysine in BSA. Block (1956) reported 49 lysine residues in CSA. Therefore, the calculated values for these proteins are reasonable.

The amino acid values of the native proteins are comparable to those reported in Alaiz and Giron (1994) for BSA and Block (1956) for CSA. The serine and glycine contents of the KLH conjugate also decreased as compared to native KLH, but did not show a significant change. Since serine and glycine do not have reactive sidechains that are susceptible to reacting with lipid oxidation products (Hall, 1987), it is highly unlikely they are being modified by hexanal.

The amino acid analysis suggests that the conjugates were highly modified. The percent modification of the hex-CSA, hex-BSA and hex-KLH conjugates was determined

Table 3.1. Amino acid content of native and hexanal modified (hex-) chicken serum albumin (CSA), bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH).

	Mole Percent[1]					
Amino Acid	Native CSA	Hex-CSA	Native BSA	Hex-BSA	Native KLH	Hex-KLH
Asp	9.20± 0.35	12.71±0.04	11.01± 0.05	12.33±0.06	13.08±0.40	13.99±0.04
Glu	15.06±0.04	17.24± 0.68	14.58± 0.11	16.47±0.11	10.75±0.97	11.81±0.08
Ser	6.84± 0.05	7.58± 0.11	4.78± 0.0	5.44±0.07	8.12±2.57	6.65±0.07
Gly	5.01± 0.10	5.34± 0.06	3.62±0.10	3.89±0.05	7.35±1.07	7.12±0.00
His	2.33± 0.06	2.44± 0.51	2.97±0.13	3.55±0.03	5.23±0.32	5.95±0.04
Arg	4.84± 0.09	5.18± 0.39	4.47± 0.06	5.00±0.32	4.62±0.35	5.10±0.12
Thr	3.70± 0.25	4.17± 0.26	5.41± 0.15	6.36±0.60	4.93±0.11	5.15±0.07
Ala	8.67± 0.33	9.21± 0.13	8.95± 0.11	9.80±0.32	7.48±0.20	7.95±0.38
Pro	6.11± 0.02	6.72± 0.07	6.61± 0.02	7.32±0.15	6.02±0.15	6.31±0.16
Tyr	3.77± 0.02	4.03± 0.07	3.71± 0.04	4.30±0.08	4.49±0.02	4.78±0.04
Val	5.79± 0.03	6.02± 0.02	5.79± 0.04	6.31±0.08	5.48±0.11	5.43±0.07
Met	2.98± 0.06	3.11± 0.04	********[2]	*********[2]	*********[2]	*********[2]
Ile	4.49± 0.02	4.61±0.05	1.90± 0.04	2.28±0.11	4.46±0.52	4.64±0.106
Leu	7.05± 0.06	7.33± 0.41	10.31± 0.01	11.14±0.14	7.86±0.38	8.65±0.18
Phe	5.27± 0.02	5.38± 0.20	4.95± 0.01	5.44±0.02	5.79±0.25	6.33±0.24
Lys	8.84± 0.02	*******[2]	10.79±0.01	********[2]	4.37±0.14	*********[2]

[1] Values are expressed as mean ± standard deviation.
[2] Values below the limit of detection.

by TNBS assay to be 95.3%, 94.8% and 86.8%, respectively. Previous amino acid analysis of the unmodified chicken serum albumin (CSA) and the hexanal modified CSA (hex-CSA) showed the available lysine content to be 11.6 and 0.2 mole %, respectively (Smith, 1997). These results indicated a 99% reduction in available lysine which corresponded to the 99% modification of the conjugate that was measured by the TNBS assay. Based on the results from both of these studies, the TNBS results were believed to be an accurate measurement of the amount of modification.

3.4.2. Hybridoma Production

Hexanal modified keyhole limpet hemocyanin (hex-KLH) and hexanal modified bovine serum albumin (hex-BSA) used for immunization of the mice were determined to have 74.6% and 97.4%, respectively, of their lysine groups modified by hexanal. All of the mice showed an immunogenic response one week after the second boost. Titers were determined by indirect ELISA using serial dilutions of the mouse sera obtained through tail bleeding 5 weeks after the initial immunization. There was a problem with the bleeding of the mice prior to immunization with the hex-BSA conjugate and not enough serum was removed from each mouse to allow for individual testing, so the sera was pooled and then tested (Figure 3.1.). The titers for the hex-BSA injected mice ranged from $4.0x10^4$ to $5.3x10^4$, 5 weeks after the initial injection (Table 3.2.). There was enough serum removed from each of the hex-KLH injected mice to allow for individual testing of the sera obtained prior to injection (Figure 3.2.) The titers for the hex-KLH injected mice were higher than the hex-BSA injected mice, ranging from $6.9x10^4$ to $9.5x10^4$ (Table 3.2.). An indirect competitive ELISA was performed using the sera from the first

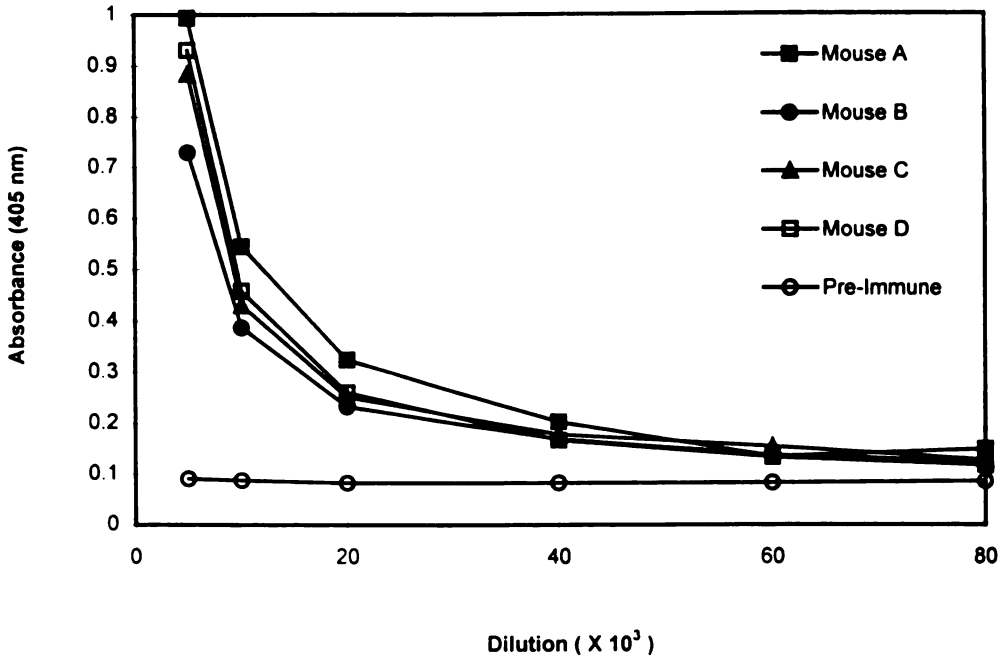

Figure 3.1. Production of antibodies against hexanal modified bovine serum albumin (BSA) was determined by indirect ELISA. Sera were obtained from the mice by tail bleeding 5 weeks after the initial injection. The initial injection and booster injections at 2 and 4 weeks contained 50 μg of hexanal modified BSA. Serum for mouse E was lost. Due to problems with pre-immune bleeding, sera from all 5 mice were pooled so as to have enough sera to perform the ELISA. Microtiter wells were coated with 100 μL of 5 μg/mL of hexanal modified keyhole limpet hemocyanin (KLH) and incubated with different dilutions of sera.

Table 3.2. Sera titers against hexanal modified bovine serum albumin (hex-BSA) or keyhole limpet hemocyanin (hex-KLH) determined 5 weeks after the initial immunization[1]

	Titer ($x10^4$)	
Mouse	Hex-BSA	Hex-KLH
A	4.0	8.7
B	4.0	7.2
C	4.6	6.9
D	5.3	8.1
E	—[2]	9.5

[1] Titers were determined 5 weeks after the initial injection. The mice received two booster injections, at week 2 and 4, with 50 μg of hexanal modified protein. The titer was arbitrarily determined to be the dilution of serum in which the absorbance is twice the absorbance of the pre-immune serum at the same dilution as measured in an indirect ELISA. Microtiter wells were coated with 100 μL of 5 μg/mL of hexanal modified protein (hexanal modified KLH was used to test BSA injected mouse sera and hexanal modified BSA was used to test KLH injected mouse sera). Plates were incubated with different dilutions of sera.

[2] Serum for mouse E was lost during storage.

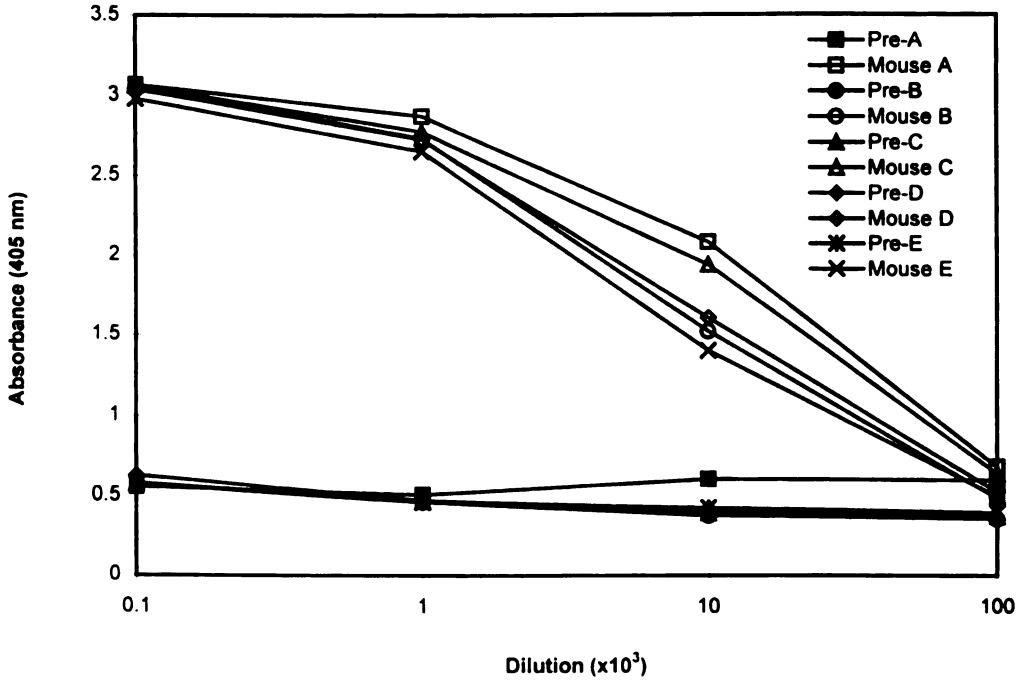

Figure 3.2. Production of antibodies against hexanal modified keyhole limpet hemocyanin (KLH) was determined by indirect ELISA. Sera was obtained from the mice by tail bleeding 5 weeks after the initial injection. The initial injection and booster injections at 2 and 4 weeks contained 50 μg of hexanal modified KLH. Microtiter wells were coated with 100 μL of 5 μg/mL of hexanal modified bovine serum albumin (BSA) and incubated with different dilutions of serum. Pre-x refers to the pre-immune sera which was removed from each mouse prior to the first injection.

bleeding of the hex-BSA injected mice and hex-BSA at concentrations of 0, 0.01, 0.1, 1, 10 and 100 μg protein/mL (Figure 3.3.). Sera from all of the mice gave greater than 80% binding inhibition at 100 μg protein/mL. This indicates that the mice were producing antibody that could recognize not only the hex-BSA attached to the plate, but also free hexanal-BSA.

Although the hex-KLH mice had higher titers, the hex-BSA injected mice were used for the fusion since the hex-BSA mice showed positive results in the indirect competitive ELISA prior to the first bleeding of the hex-KLH injected mice. Mouse B and mouse D were selected for the fusion because their sera showed the lowest recognition of native BSA when tested in an indirect ELISA. However, mouse A might have been a better choice than mouse D because serum from mouse A gave higher binding inhibition in the competitive ELISA. At 10 μg protein/mL, mouse A gave a binding inhibition of 73% whereas mouse D was 46%. In the indirect ELISA for determining recognition of the native BSA, at 1:1000 serum dilution, mouse D had an absorbance of 0.694 while mouse A had an absorbance of 1.087. However, at the next higher dilution, 1:3160, the absorbances for mouse D and A were 0.298 and 0.493, respectively. This is a small difference in the recognition of the native protein by the sera from these mice, so the indirect competitive ELISA results were probably a better indication of antibody production..

Following the fusion, the cells were plated out and allowed to grow. The fusion efficiency (the number of wells showing growth/number of wells seeded) of the plates was determined and found to be 89% (425/480). Eight positive hybridomas were

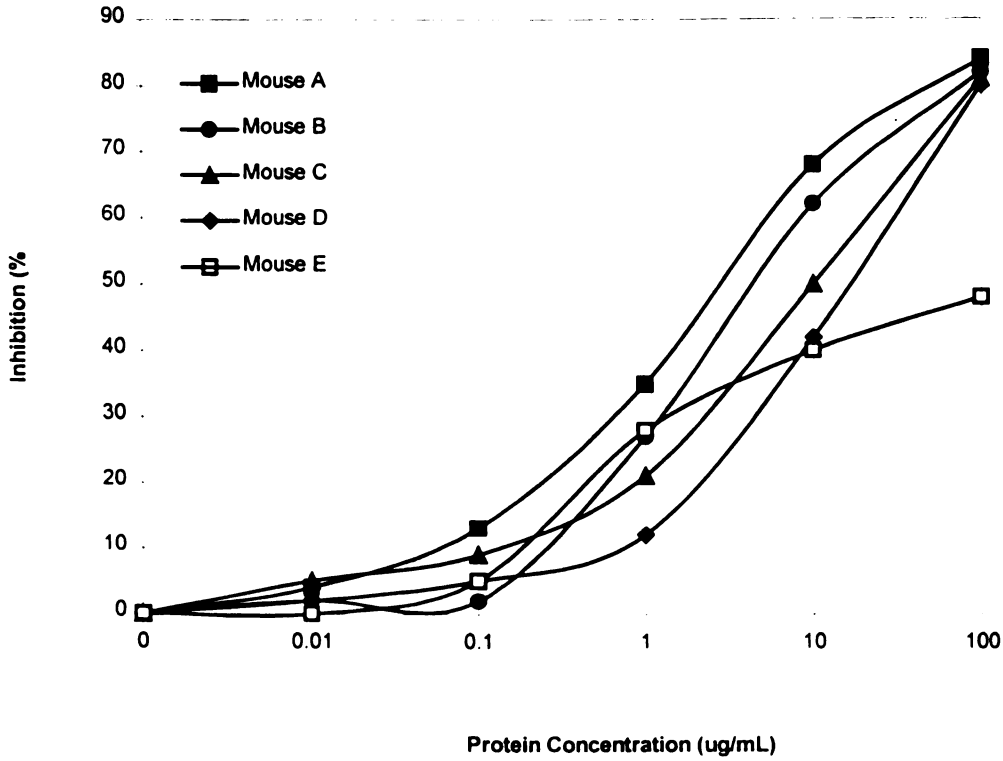

Figure 3.3 Antibody production by hexanal modified bovine serum albumin (hex-BSA) injected mice was determined by indirect competitive ELISA, using serum from the first bleeding. Mice were tail bled five weeks after being injected with 50 μg hex-BSA. Boosters of 50 μg hex-BSA were given at weeks 2 and 4. Microtiter wells were coated with 100 μL of hex-BSA (5 μg protein/mL). A 1/1000 dilution of sera was incubated with free hex-BSA at concentrations of 0.01, 0.1, 1, 10 and 100 μg/mL of BSA.

produced from both fusions and 3 were further cloned. The hybridoma which showed the highest binding inhibition in the indirect competitive ELISA from the first cloning was cloned a second time. The hybridoma from the second cloning showing the highest binding inhibition and the least cross-reactivity with aliphatic aldehydes other than hexanal was identified and designated as 45P1-D9. The isotype of this clone was IgG_1. The monoclonal antibody secreted by this cell line was used for the optimization of the indirect competitive ELISA, as well as for the epitope studies.

3.4.3. Protein Specificity

The antibody was first checked, by indirect competitive ELISA, for recognition of free hexanal. There was no recognition of free hexanal in 30% methanol at concentrations up to 1000 ng hexanal/mL. Methanol was added to help make the free hexanal more soluble. This concentration of methanol had been previously determined not to cause interference with the ELISA using a polyclonal antibody (Smith, 1997) and was found not to cause interference with the monoclonal antibody ELISA.

After determining that the antibody did not recognize free hexanal, it was checked for recognition of hexanal conjugated to different carrier proteins. The degrees of modification for the hex-CSA, hex-BSA and hex-KLH were 99.1%, 95.3% and 90.9%, respectively. There was less than 5% binding inhibition with 5 μg protein/mL of the native protein (CSA, BSA, and KLH dissolved in PBS; pH 7.2) or the protein blank (CSA, BSA and KLH that had been through the conjugation reaction but without any hexanal added). At 10 μg protein/mL, the hex-CSA conjugate showed 88% binding

inhibition, the hex-BSA conjugate showed slightly less inhibition, 78%, and the hex-KLH conjugate showed only 42% (Figure 3.4.) The difference in the amount of binding inhibition for each of the conjugates, at the same protein concentration, is partly due to the moles of lysine in each protein. Since hexanal binds to the ε-amino group of lysine, the protein with the most available lysine should bind the most hexanal. Native KLH was determined, by amino acid analysis, to have 4.37 mole % lysine as compared to 8.84 and 10.79 mole %, respectively, for CSA and BSA. Both BSA and CSA have more than twice the number of lysine residues as KLH. Also, KLH has a large molecular weight, compared to BSA and CSA and as a result, there are fewer moles of KLH at the same protein concentration as BSA and CSA, as well as fewer lysines.

The hexanal concentration of the modified proteins was calculated for each protein concentration, using the moles of lysine and percent modification. The calculated moles of lysine per mole of protein were 580, 56 and 46 for KLH, BSA and CSA, respectively. Although the difference in percent modification and the moles of lysine had been adjusted for, the protein conjugates gave different binding inhibitions at the same hexanal concentration (Figure 3.5.) At 8 ng hexanal/mL, binding inhibition for the KLH, BSA and CSA conjugates were 7, 16 and 19%, respectively. This shows that the type of protein has an effect on the binding inhibition. This may be due to the antibody showing some recognition of the carrier protein. Since the mice were immunized with hex-BSA and hex-KLH, hex-CSA was used as the competitor in the screening assay. As a result, the antibodies selected may show a preference for the hex-CSA conjugate. Although the

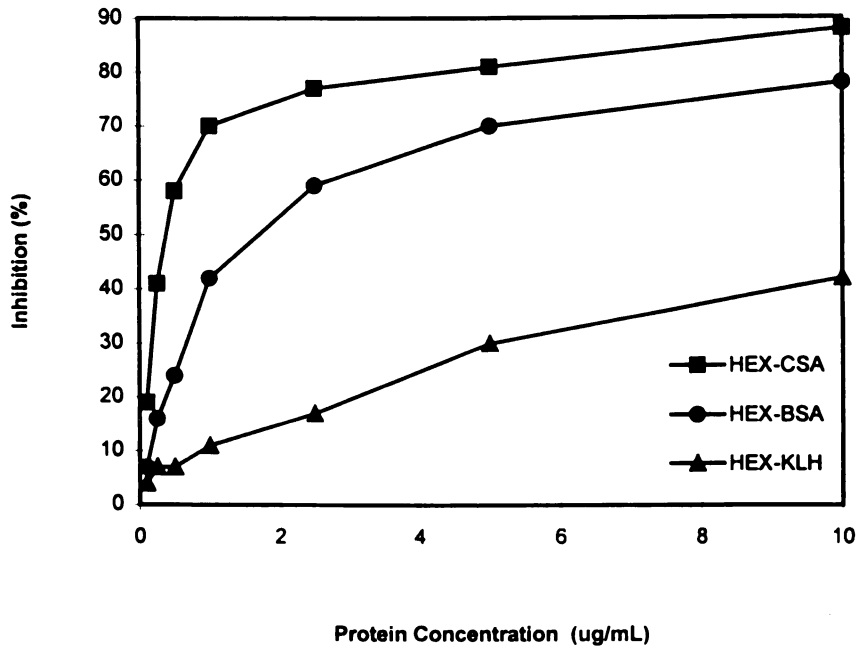

Figure 3.4. Specificity of monoclonal antibody to hexanal modified proteins was determined by indirect competitive ELISA. Microtiter wells were coated with 1 μg/mL of hexanal modified chicken serum albumin (hex-CSA). A 1/1000 dilution of antibody (1.6 μg/mL) was incubated with competing native or hexanal modified CSA, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) at concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg/mL protein concentration. Each data point represents 6 replicates. The amount of modification for BSA, CSA and KLH, as determined by TNBS assay, was 95.3%, 99.1% and 90.9% respectively. The standard error of the means, at all concentrations, were 0.63, 0.78 and 1.43 for hex-CSA, hex-BSA and hex-KLH, respectively.

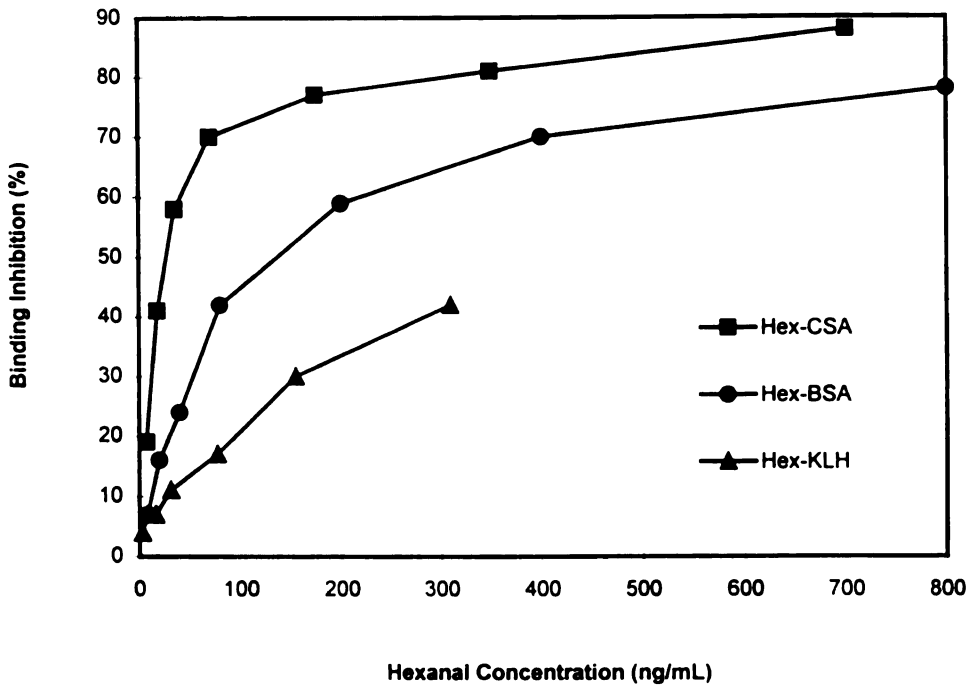

Figure 3.5. Effect of hexanal concentration on the binding inhibition of hexanal modified proteins was determined by indirect competitive ELISA. Microtiter wells were coated with 1 μg/mL of hexanal modified chicken serum albumin (hex-CSA). A 1/1000 dilution of antibody (1.6 μg/mL) was incubated with competing hexanal modified CSA, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) conjugates at concentrations of 0 to 800 ng hexanal/mL. Each data point represents 6 replicates. The amount of modification for BSA, CSA and KLH, as determined by TNBS assay, was 95.3%, 99.1% and 90.9% respectively.

antibodies may preferentially bind to hex-CSA, nevertheless, they do not recognize native CSA, indicating the antibody is recognizing the hexanal attached to CSA, not CSA.

Sodium cyanoborohydride was added to reduce the double bond that is formed by the Schiff base reaction between the lysine side chains of the protein and hexanal. It was also determined by indirect competitive ELISA that the antibody recognized only the conjugates that have had this bond reduced. Klassen et al. (1994) found their monoclonal antibody to acetaldehyde adducts also recognized only reduced conjugates.

These results suggest that the antibody does not recognize free hexanal but recognizes hexanal modified proteins, regardless of the protein carrier. These results are consistent with the results found using polyclonal antibodies for hexanal (Smith, 1997). Polyclonal antibodies did not recognize free hexanal, but needed the protein carrier. Studies using monoclonal antibodies to acetaldehyde adducts have also reported similar results. Israel et al. (1986) developed an antibody to acetaldehyde which recognized the acetaldehyde modified erythrocytes, but not the unmodified erythrocytes. Klassen et al. (1994) found their monoclonal antibody recognized acetaldehyde conjugated to BSA, KLH or actin, but did not recognize unreduced conjugates or unmodified proteins.

3.4.4. ELISA Optimization

The hexanal-CSA conjugate was used to optimize the ELISA because it was the most highly modified and gave the best binding inhibition over a range of concentrations. Protein concentrations used in the standard curve were converted to hexanal concentration as described in the methods and materials. A representative standard curve for the indirect competitive ELISA that was used for the epitope studies shows the

working range was 1 to 50 ng hexanal/mL (Figure 3.6.). The limit of detection for this assay was 1 ng hexanal/mL. The limit of detection of the polyclonal antibody ELISA was determined to be 7.4 ng/ml (Smith, 1997), with a working range of 7.4-740 ng hexanal/mL. With a working range of 1 to 50 ng/mL, the monoclonal based ELISA is more sensitive for hexanal conjugates than the polyclonal antibody assay used by Smith (1997).

The reproducibility of the ELISA, within and between assays, was also determined (Table 3.3.). Deshpande (1996) recommended using at least 3 analyte concentrations that represent low (80% B_O), midrange (50%B_O) and high (20% B_O) binding inhibition when evaluating precision. B_O is equal to the binding of the zero standard, or maximum binding. For this ELISA format, 5ng/mL and 40 ng/mL represented the low and midrange binding, respectively. A higher concentration that was equal to 80% B_O was not used because it was outside of the working range of the assay (1 to 50 ng/mL). The 10 and 20 ng/mL concentrations were used for a broader range of concentrations within the working range of the standard curve. The coefficient of variation (%CV) ranged from 4.15 to 6.43 for the intra- assay variability and from 5.07 to 7.71 for inter-assay variability.

3.4.5. Cross-reactivity Study

The cross-reactivity of the antibody with other aliphatic aldehydes was determined using CSA conjugates in an indirect competitive ELISA. CSA was modified as outlined in section 3.3.2. with different aldehydes and the extent of modification was

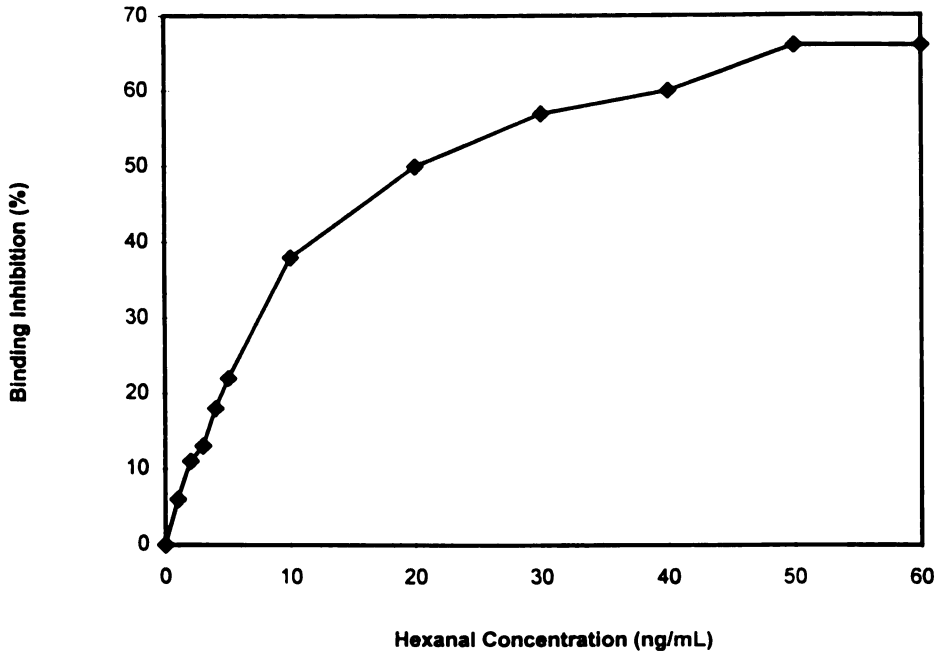

Figure 3.6. Representative standard curve showing the working range of the indirect competitive ELISA for hexanal. The working range was determined to be 1 to 50 ng hexanal/mL. Each data point represents 8 replications. Microtiter wells were coated with 100 µL of 1ug/mL hexanal modified CSA. A 1/1000 dilution of antibody (1.6 µg/mL) was incubated with hex-CSA at concentrations of 0, 1, 5, 10 and 50 ng/mL of hexanal. The standard error of the means was 0.869 for all concentrations except for 40 ng/mL, which was 0.929.

Table 3.3. Reproducibility of the indirect competitive ELISA was determined for both within a run (intra-assay) and between runs (inter-assay).

Concentration[2] (ng hex/mL)	Coefficient of Variation[1]	
	Intra-assay[2] (%)	Inter-assay[3] (%)
0	4.40	5.07
5	5.60	5.28
10	6.22	6.56
20	6.43	7.71
40	4.15	7.31

[1] Coefficient of variation was calculated by dividing the standard deviation of the data series by the mean and multiplying by 100.

[2] Intra-assay variability was calculated using 16 replicate wells.

[3] Inter-assay variability was calculated using 32 replicates from eight different plates.

Table 3.4. Loss of reactive amino groups in CSA solutions was measured by trinitrobenezesulfonic acid (TNBS) assay

Solution	Loss (%)[1]
Propanal-CSA	96.1
Butanal-CSA	97.3
Pentanal-CSA	97.2
Hexanal-CSA	97.2
Heptanal-CSA	97.3
Octanal-CSA	97.4
Nonanal-CSA	97.4
2-*t*-hexenal-CSA	81.4
2-methylpentanal-CSA	96.1
2-methylbutanal-CSA	96.0
3-methylpentanal-CSA	96.0

[1] Defined as percentage decrease in TNBS reactive amino groups of native protein.

expressed as percent loss of reactive amino groups (Table 3.4.) The antibody did not cross-react with the 3 (propanal), 4 (butanal) and 9 (nonanal) carbon aldehydes when conjugated to CSA (Figure 3.7.). There was less than 8% binding inhibition at 100 ng aldehyde/mL of these three conjugates. Although there was some recognition of the 8 (octanal) carbon aldehyde, the antibody did not cross-react with it because the binding inhibition did not reach 50%. Antibody cross-reactivity with the 5 (pentanal) and 7 (heptanal) carbon aldehyde-protein conjugates was 37.9% and 76.6%, respectively (Table 3.5.). Since all of these aldehyde conjugates showed greater than 90% modification, the differences in cross-reactivity were caused by differences in antibody recognition and not by a difference in the extent of modification.

The antibody also cross-reacted (45.0%) with 2-*t*-hexenal when conjugated to CSA (Table 3.5.). 2-*t*-Hexenal is a six carbon aldehyde with a double bond between the second and third carbons. Jentoff and Dearborn (1979) demonstrated that $NaCNBH_3$ could be used to reduce the double bonds in Schiff bases. The double bond of 2-*t*-hexenal may also be reduced, along with the Schiff base formed between 2-*t*-hexenal and the lysine residues of the protein, in the presence of $NaCNBH_3$. This would result in the formation of hexanal during the conjugation step. The lower cross-reactivity of the antibody with this conjugate may be due to incomplete reduction of the double bond to form hexanal. The lower cross-reactivity may also be due to lower lysine modification (87% as measured by TNBS) of the protein, resulting in fewer moles of aldehyde in the conjugate. Smith (1997) determined that the polyclonal antibody to hexanal cross-reacted 100% with a 2-*t*-hexenal conjugate. The conjugate was determined to be 96% modified,

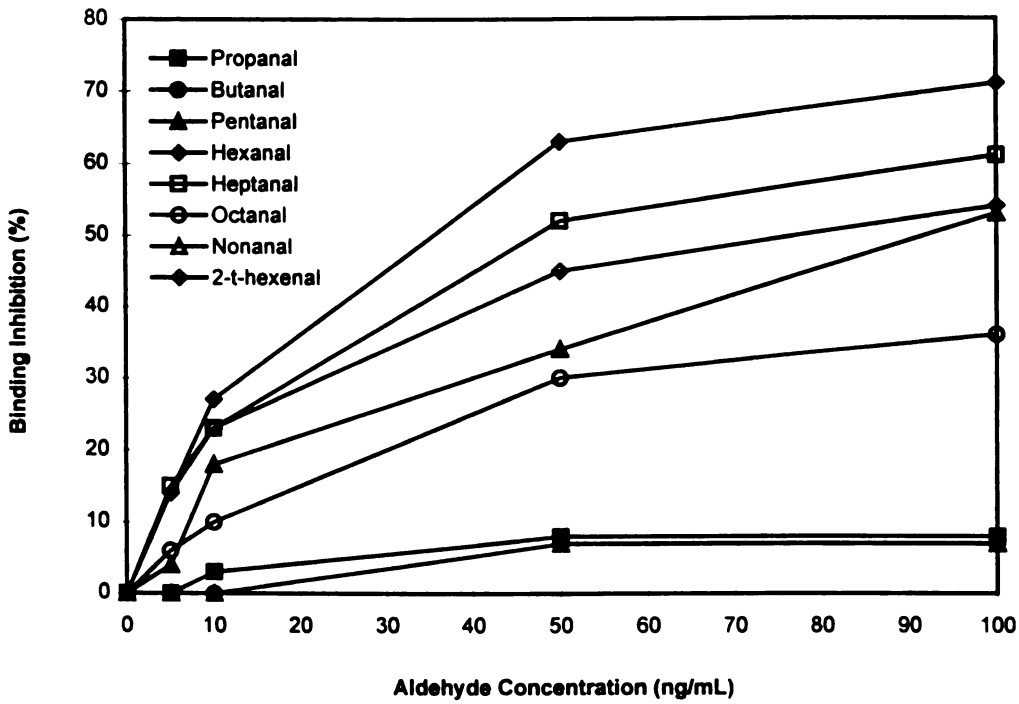

Figure 3.7. Specificity of the monoclonal antibody to aliphatic aldehydes conjugated to chicken serum albumin (CSA). Each data point represents 4 replications. Microtiter wells were coated with 100 µL of 1µg/mL hexanal modified chicken serum albumin (hex-CSA). A 1/1000 dilution of antibody (1.6 µg/mL) was incubated with aldehdye-CSA conjugates at concentrations of 0, 5, 10, 50 and 100 ng aldehyde/mL. The standard error of the means, at all concentrations, were 2.21, 1.52,0.68, 1.09, 0.73, 0.76, 1.10 and 0.57 for the propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal and 2-*t*-hexenal CSA conjugates, respectively, at all concentrations.

Table 3.5. Cross-reactivity of the monoclonal antibody to aldehyde modified chicken serum albumin (CSA) by indirect competitive ELISA.

Aldehyde Structure	Conjugate	Cross-Reactivity[1] (%)
Hexanal	CSA-$NH(CH_2)_5CH_3$	100
Heptanal	CSA-$NH(CH_2)_6CH_3$	76.7
2-*t*-hexenal	CSA-$NHCH_2CH{=}CH(CH_2)_2CH_3$	45.0
Pentanal	CSA-$NH(CH_2)_4CH_3$	37.9

[1] Defined as the concentration of hexanal-CSA required for 50% inhibition of binding divided by the concentration of aldehyde-CSA required for 50% inhibition of binding multiplied by 100.

by TNBS assay, indicating the low cross-reactivity of this monoclonal antibody to the 2-*t*-hexenal conjugate may be due to lower modification.

Two compounds, 2-methylbutanal and 2-methylpentanal, are Strecker degradation products. The Strecker degradation pathway involves the interaction of α-dicarbonyl compounds with α-amino acids resulting in volatile aldehydes, pyrazine and sugar fragments. This reaction often occurs during cooking of meat. These compounds, as well as a structurally related compound, 3-methylbutanal, and a 7 carbon ketone (2-heptanone) were also checked for cross-reactivity. There was less than 5% binding inhibition at 100 ng/mL concentration of 2-methylbutanal and 3-methylbutanal conjugates, and only slightly more binding inhibition of the 2-methylpentanal conjugate (9% inhibition of binding at 100 ng/mL concentration). The low binding inhibition indicates that conjugates made with branched aldehydes were not recognized by the antibody. The antibody showed no recognition of the 2-heptanone conjugate (95.9% modified).

CSA was also modified by n- and 2-hexanol. The TNBS assay showed the lysine residues of the n-hexanol-CSA and 2-hexanol-CSA conjugates to be 78.0% and 77.0% modified, respectively. Although it was theorized that the alcohols might be reduced to aldehydes during the conjugation procedure, there was less than 8% binding inhibition at 100 ng alcohol/mL. The reason may be due to using sodium cyanoborohydride as a reducing agent. Sodium cyanoborohydride is a weak reducing agent that readily reduces Schiff bases, but does not reduce aldehydes (Jentoft and Dearborn, 1979). Sodium cyanoborohydride, therefore, may not be strong enough to reduce the alcohols to aldehydes.

These results indicate that the antibody is most specific to the straight chain, 6 carbon aldehyde, when conjugated to CSA. These results are similar to results obtained by Smith (1997), where the cross-reactivity of the polyclonal antibody was found to increase as the carbon number in the aliphatic aldehyde approached six. Although the polyclonal antibody did cross-react with the branched aldehyde and ketone conjugates, the amounts were small (less than 1.1%). The results indicated that the polyclonal antibody was also most specific to 6 carbon aliphatic aldehydes, conjugated to CSA.

The next part of the study was designed to determine if the monoclonal antibody could recognize hexanal when conjugated to amino acids. The proposed epitope recognized by the monoclonal antibody is the lysine-hexanal complex, based on previous work by Smith (1997) and that this antibody does not recognize free hexanal. Hexanal was conjugated to lysine, glycine and arginine, as well as ε-amino caproic acid, a lysine derivative, to see what functional groups were required for recognition by the antibody. The TNBS assay showed the available reactive sites on the lysine, glycine, arginine and ε-amino caproic conjugates to be 97.6%, 97.4%, 97.5% and 97.4% modified, respectively, by hexanal. There was less than 3% binding inhibition at 2 mM amino acid concentration with the hexanal modified glycine, arginine and the unmodified amino acids. The antibody showed the highest recognition of the hexanal modified ε-amino caproic acid. At the 2 mM concentration, the ε-amino caproic acid conjugate caused a 46% binding inhibition while the hexanal modified lysine conjugate showed 17% binding inhibition at the same concentration (Figure 3.8.). This was as expected since ε-amino caproic acid has only the ε amino terminal group available to bind hexanal and is

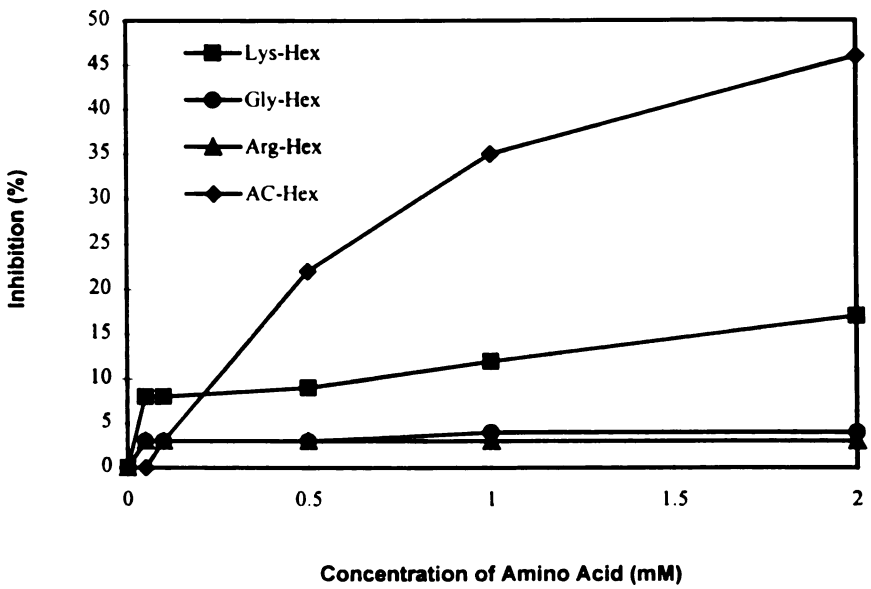

Figure 3.8. Specificity of the monoclonal antibody to hexanal modified amino acids was determined by indirect competitive ELISA. Each data point represents 4 replications. Microtiter wells were coated with 100 μL of hex-CSA (1 μg protein/mL). A 1/1000 dilution of antibody (1.6 μg/mL) was incubated with 0, 0.05, 0.1, 0.5, 1 and 2 mM amino acid. The standard error of the means, at all concentrations, were 2.31, 1.82, 1.77 and 5.51 for hexanal modified lysine, glycine, arginine and amino caproic acid, respectively, at all concentrations.

representative of the lysine side chains found in protein. The free lysine has both the α and ε amino groups available for binding to hexanal and may form a conjugate the antibody cannot recognize. It is possible that the antibody does not recognize hexanal bound to only the α amino group of lysine or if hexanal is bound to both the α and ε amino groups.

Our results are similar to those of other researchers. Smith (1997) also found that the ε-amino caproic acid conjugate was a better competitor in the polyclonal ELISA for hexanal than the lysine conjugate was. Thiele et al. (1997) characterized a monoclonal antibody that recognized acetaldehyde adducts formed with *N*-ethyl lysine. The cross-reactivity of the antibody with acetaldehyde adducts formed with other amino acids (arginine, ethylamine and lysine) was determined; however, this antibody was found to be specific to only the acetaldehyde adduct formed with *N*-ethyl lysine. The antibody used in our study also appears to be specific to only one adduct, the hexanal modified lysines within a protein chain.

These results indicate that this monoclonal antibody was most specific to the hexanal modified lysine side chain of proteins. The ELISA developed using this antibody was sensitive to 1ng hexanal/mL. Based on its specificity and sensitivity, this ELISA shows promise for being useful in actual systems. Although the ELISA will detect some pentanal and heptanal, both these compounds are lipid oxidation products. The ELISA could be used to detect not just hexanal, but to monitor lipid oxidation by measuring all three products.

Chapter 4

VERIFICATION OF A MONOCLONAL ANTIBODY BASED ELISA FOR MONITORING LIPID OXIDATION BY CORRELATION TO GAS CHROMATOGRAPHY

4.1. Abstract

An indirect competitive (IC) ELISA for monitoring lipid oxidation was compared with a commonly used gas chromatography (GC) method by measuring the hexanal concentration of chicken serum albumin (CSA) that was differentially modified with hexanal. The conjugates were tested in the ELISA at 3 dilutions (1/500, 1/1000 and 1/2000) and all 3 dilutions correlated well with the GC method (r = 0.975, 0.952 and 0.978, respectively). Native and hexanal modified proteins extracted from raw chicken breast were separated by SDS-PAGE and used in a western blot. The antibody recognized only the hexanal modified proteins. A frozen storage study was conducted using raw, ground chicken meat. The hexanal concentration, as measured by IC-ELISA, peaked at 3 months with 3.3 μg hexanal/g breast meat and 4.02 μg hexanal/g thigh meat. Although hexanal concentration by GC increased, the concentration was still low even after 9 months of storage. The breast contained only 0.42 μg hexanal/g meat and the thigh contained 2.05 μg hexanal/g meat. A refrigerated storage study was conducted using

ground, cooked chicken. An alkaline protease was used as an aid in solublizing the cooked chicken proteins. The maximum hexanal concentration, by GC, was 12.41 μg/g of thigh meat after 60 h storage and 7.06 μg/g of breast meat after 3 days storage. Using the IC-ELISA, the concentration of hexanal in breast meat did not change, whereas the concentration of the thigh reached a maximum of 1.41 μg hexanal/g meat after 3 days. Although the study with the differentially modified protein showed the IC-ELISA and GC to correlate well, the storage studies with the chicken muscle were inconclusive. Further studies of the reactions occurring between hexanal and meat proteins need to be conducted so the IC-ELISA may be further optimized for use with a meat system.

4.2. Introduction

Oxidation plays a major role in consumer acceptance of muscle foods. Oxidation can affect a range of meat components including proteins, lipids, vitamins, carbohydrates and pigments (Kanner, 1994) resulting in changes in color, flavor and nutritive value of the meat (Love and Pearson, 1971). Intrinsic factors can have an effect on the extent of oxidation. Rhee et al. (1996) found the higher the concentration of heme iron and unsaturated fatty acids in a meat cut, the more likely the meat was to oxidize. Extrinsic factors, such as cooking or storage temperature, can also influence oxidation. Cooked meats develop rancid odors and flavors faster than raw meat (Ladikos and Lougovois, 1990).

The 2-thiobarbituric acid (TBA) is the most commonly used method for monitoring lipid oxidation in foods. Two moles of TBA react with one mole of

malonaldehyde (MA) to form a pink complex that is measured spectrophotometrically at 532 nm (Botsoglou et al., 1994). TBA results are expressed as milligrams of MA per kilogram of sample (Melton, 1983). The TBA test is now often referred to as the thiobarbituric acid reactive substances (TBARS) test because it lacks specificity to MA. As a result of this lack in specificity, TBARS has been widely criticized. Gray and Monahan (1992) suggest using the TBARS test, but to keep in mind its limitations and use a second method, such as hexanal concentration, for monitoring lipid oxidation.

Hexanal is a six carbon aldehyde that is the major secondary product formed when linoleic acid is oxidized (Gray and Monahan, 1992). Hexanal concentration is generally measured using gas chromatography (GC). The most commonly used GC method is head space (HS) concentration (Reineccius, 1996). This method involves purging the sample with an inert gas and trapping the volatiles. The volatiles are removed from the trap by heating (thermal desorption) or by using a solvent (chemical desorption) prior to injection into the GC. Hexanal concentration, as measured by HS-GC, has been shown to correlate with sensory scores and TBARS numbers for cooked pork (Shahidi et al., 1987), cooked beef (St. Angelo et al., 1987) and restructured chicken nuggets (Lai et al., 1995).

Although GC is widely used, this method has several drawbacks including the need for dedicated equipment, inconvenience of analyzing only one sample at a time and possible peroxide decomposition to additional volatiles from the heat used to drive volatiles onto the column (Ajuyah et al., 1993). An alternative to GC is an enzyme linked immunosorbent assay (ELISA) for determining hexanal concentration. A monoclonal antibody against hexanal was developed and an ELISA devised (Chapter 3, section

3.3.5.). The antibody was characterized to determine the usefulness of the ELISA. The antibodies recognized hexanal conjugated to bovine serum albumin (BSA), chicken serum albumin (CSA) and keyhole limpet hemocyanin (KLH) but did not recognize free hexanal, native proteins or free amino acids. The antibody cross-reacted with pentanal, heptanal and 2-*t*-hexenal (37.9%, 76.6% and 45.0%, respectively) conjugated to CSA, as well as reacting strongly with hexanal modified amino caproic acid. Based on these results, we proposed that the antibody recognized the hexanal-lysine complex formed via Shiff base reactions.

Although the antibody cross-reacted with pentanal, heptanal and 2-*t*-hexenal conjugates, as well as recognizing hexanal conjugates, this should not interference with the usefulness of the ELISA. Pentanal and heptanal have much lower odor thresholds than hexanal (Brewer and Vega, 1995), and are also important contributors to oxidized flavors. The ELISA could be used to monitor lipid oxidation by measuring the concentration of not just hexanal, but also pentanal and heptanal.

Another indirect ELISA for monitoring hexanal concentration, using this same monoclonal antibody, was devised by Smith (1997). The ELISA was used, along with a GC method and a TBARS assay, to monitor lipid oxidation in a model meat system. Hexanal concentration measured by that monoclonal ELISA was found to correlate with hexanal concentration by GC and TBARS numbers ($r = 0.81$ and 0.77, respectively).

The purpose of this study was to determine if hexanal concentrations quantified by the monoclonal antibody based ELISA devised in the previous chapter would correlate with hexanal concentrations measured by a common GC method. Chicken serum albumin was modified with different amounts of hexanal and the hexanal

concentration of the conjugates, measured by ELISA and GC, was correlated. Extraction procedures for raw and cooked meat were devised. Storage studies were then designed to see if the hexanal concentration in an actual meat system, measured by GC and ELISA, could be correlated.

4.3. Methods and Materials

4.3.1. Materials

The goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (IgG-HRP) was purchased from Cappel Laboratories (West Chester, PA). The 60/80 mesh Tenax-TA was purchased from Alltech Associates, Inc. (Deerfield, IL) and the silane treated glass wool was from Supelco, Inc. (Bellefonte, PA). The 10.2 cm long metal traps were from MSU Biochemistry Shop (East Lansing, MI). The solvent, 2-methyl butane, was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Whirl Pak bags were from Nasco (Fort Atkinson, WI) and resealable storage bags were from Gorden Food Service (Grand Rapids, MI). Enzeco® Alkaline Protease-L FG (minimum enzyme activity was 560,000 Delft units per gram, standardized with glycerol) was from Enzyme Development Corporation (New York, NY). Chicken was purchased from local retailers. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

4.3.2. Hexanal Modification of CSA

Chicken serum albumin (CSA) was modified with different amounts of hexanal as described in Chapter 3, section 3.3.2., except that $NaCNBH_3$ was not used when

preparing samples for GC analysis. Briefly, 0.62, 1.23, 2.46, 6.15, 12.3, 18.5, 24.6, 37.0 or 49.2 μL of hexanal were added to 8 mL of CSA (12.5 mg/mL) diluted in phosphate buffered saline (PBS; 0.1M sodium chloride, 0.01M sodium phosphate, pH 7.4). The volume was brought up to 9 mL with PBS (pH 7.4). Half of this solution (4.5 mL) was mixed with 0.5 mL of PBS (pH 7.4) and used for gas chromatography. The final reactant concentrations were 10 mg/mL of protein and 0.5, 1, 2, 5, 10, 15, 20, 30 and 40 mM of hexanal. The other half (4.5 mL) of the solution was prepared for ELISA by adding 0.5 mL of sodium cyanoborohydride, diluted in 0.1N NaOH, to a final concentration of 20 mM sodium cyanoborohydride. The final reactant concentrations of the protein and hexanal were the same as the GC samples. The reaction was allowed to proceed overnight. The percentage of protein modification of the conjugates used in the ELISA was determined using a trinitrobenzenesulfonic acid (TNBS) assay (Habeeb, 1966) as modified by Smith (1997).

4.3.3. Hexanal Concentration by CI-ELISA

The hexanal concentration of the conjugates was determined by indirect competitive (IC) ELISA as described in Chapter 3, section 3.3.5. A standard curve was generated using hexanal-CSA at concentrations of 0 to 500 ng hexanal/mL. The differentially modified protein conjugates were diluted 1/2000, 1/1000 and 1/500 in PBS (7.2) prior to ELISA analysis. These dilutions were equivalent to 5, 10 and 20 μg protein/mL, respectively. Hexanal concentration was expressed as milligram of hexanal per gram of protein. Six replicates were performed for each concentration.

4.3.4. Isolation and Concentration of Volatiles for Gas Chromatography

Prior to analysis by GC, the volatiles present in the sample, including hexanal, were removed using a purge and trap apparatus. Volatiles from the samples were absorbed onto Tenax and desorbed using 2-methylbutane following the methods of Liu et al. (1992) and Koelsch et al. (1991) as modified by Smith (1997). Briefly, the 10.2 cm long metal traps were packed with 200 mg of 60/80 mesh Tenax-TA and the ends plugged with silane treated glass wool. The traps were conditioned for 24 h with nitrogen gas at a flow rate of 25 mL/min at 150°C. After cooling, the traps were attached to a purge and trap apparatus. For the determination of hexanal concentration in the pure protein system (CSA), 1 mL of the solution and 50 mL of water were added to a round bottomed, 500 mL flask. In the storage studies, 10 g of chicken and 50 mL of water were used. The flask was then heated at 60°C for 90 min while sparging with a 30 mL/min stream of nitrogen. To remove the volatiles from the Tenax, 2-methylbutane was added dropwise and eluted with centrifugation at 80 x g for 5 min intervals until 1.5 to 2 mL eluent was collected. The extract was then concentrated under nitrogen to 0.5 mL and stored at -20°C.

4.3.5. Hexanal Concentration by Gas Chromatography

Hexanal concentration was determined using a Hewlett-Packard 5890A gas chromatograph with a flame ionization detector. The volatiles were separated on a DB-225 fused silica capillary column (30m x 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific Inc., Rancho Cordova, CA). Samples (5 μL) were injected and run according to

the program used by Smith (1997) except that the helium flow rate was increased to 50 mL/min and a split ratio of 66 was used. Hewlett-Packard 3365 Series II Chem Station Software (Minneapolis, MN) was used for peak integration. Peak retention times of hexanal, pentanal and heptanal references were used to identify elution peaks. The results were calculated as milligrams of hexanal per gram of CSA. Each concentration of differentially modified conjugate was tested in triplicate.

4.3.6. Statistical Analysis

Correlations between the hexanal concentration, measured by GC and each protein concentration used in the ELISA, were established using linear regression analysis (SAS, SAS Institute, Inc., Cary, NC).

4.3.7. Western Blot of Hexanal Modified Salt Soluble Proteins

Boneless, skinless chicken breast meat, purchased from a local retail store, was ground twice with a Hobart Kitchen-Aid food grinder (Model KF-A, Troy, OH) using a 4 mm plate. The ground muscle was then homogenized with 3 volumes of 0.6 M NaCl; 50 mM sodium phosphate buffer using a Polytron homogenizer (Model PT 10/35; Brinkmann Instruments Co., Westbury, NY). After straining the mixture through two layers of cheesecloth, the proteins were modified with hexanal, using the method described in Chapter 3, section 3.3.2. The final reactant concentrations were 200 mM hexanal, 15 mg/mL of protein and 20 mM sodium cyanoborohydride. Both modified and unmodified chicken proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a Mini-Protean II

electrophoresis unit (Bio-Rad Laboratories, Richmond, CA). The stacking and separating gels were 4 and 12% acrylamide, respectively, with 45μg of protein loaded in each lane. Protein bands were stained with Coomassie Brillant Blue R 250. The western blot was performed as described by Wang et al. (1992) except casein was used in place of BSA for blocking and goat anti-mouse IgG peroxidase conjugate was used in place of the goat anti-rabbit conjugate. Briefly, the proteins were transferred from an unstained SDS-PAGE gel to a nitrocellulose membrane using a Mini Trans-Blot unit (Bio-Rad). After transferring, the membrane was washed and blocked with 3% casein in phosphate buffer saline (PBS; pH 7.2). After blocking, the membrane was again washed and monoclonal antibody against hexanal (1/1000) was added and allowed to incubate for 30 min. The unbound antibody was removed by washing and goat anti-mouse IgG peroxidase (1/2000) was added and again incubated for 30 min. The membrane was washed a final time and the bound peroxidase determined with 2,2' azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) substrate. The staining was stopped with water.

4.3.8. Proximate Analysis of Chicken

Protein, moisture and fat content of the raw and cooked chicken used in the storage studies was determined in triplicate following AOAC methods (1990) 981.10, 950.46 B and 991.36, respectively.

4.3.9. Frozen Storage Study (Preliminary Experiments)

Boneless, skinless chicken thighs and breasts were purchased from a local retail store. The muscle was ground twice with a Hobart Kitchen-Aid food grinder (Model KF-A, Troy, OH) using a 4 mm plate. Salt (1% of total meat weight) was dissolved in 10% added water, mixed into the ground muscle and ground twice. Meat (25 g) was placed in 24 oz. Whirl Pak bags and stored at -20°C for up to 9 months. The zero time samples were tested prior to freezing. Six bags (3 bags of each muscle) were removed at intervals and allowed to thaw at 4°C overnight. Meat from each bag was divided into two 10 g portions for testing by ELISA and GC.

The hexanal concentration was determined by GC as described above. Meat for ELISA analysis was extracted in 90 mL of 0.6 M NaCl buffer with 50 mM sodium borate (pH 9) by stirring for 1 h on a stir plate. The samples were reduced with sodium cyanoborohydride (20 mM final concentration) dissolved in 0.1N NaOH and left overnight at ambient temperature. The mixture was strained through two layers of cheesecloth, diluted 1:80 in PBS (pH 7.2) and tested by the ELISA method described above. The protein concentration of the meat extracts used in the ELISA was about 250 μg protein/mL. Hexanal concentration by both ELISA and GC was expressed as micrograms hexanal per gram of meat.

4.3.10. Optimization of Extraction Procedure for Cooked Thigh Meat

Boneless chicken thighs were ground once with a Hobart Kitchen-Aid food grinder (Model KF-A, Troy, OH) using a 4 mm plate. After grinding, 30 g meat was

placed into a 50 mL centrifuge tube and held in a circulating water bath (Model 1268-52, Cole-Parmer, Chicago, IL) at 73°C. A thermocouple (RTD, 1.6 mm diameter ± 0.5°C) connected to a Solomat MPM 2000 Modumeter (Stanford, CT) was placed in one tube containing meat. When the temperature of the meat reached 71°C, the tubes were removed and placed in ice water for 15 min. After cooking, the meat was reground twice.

An enzyme solution was prepared by diluting 25 μL of the alkaline protease in 50 mL of the extraction buffer (0.6 M sodium chloride, 50 mM sodium borate, pH 9.0). The enzyme concentration necessary to maximize protein solubility was determined by adding 2.4, 4.8, 24 or 48 μL of the enzyme solution (which corresponded to 0.05, 0.1, 0.5 and 1% (w/w) of the protein concentration) to 3 g cooked chicken and 27 mL extraction buffer (pH 9.0) in a 50 mL centrifuge tube. After shaking for 30 s, the tubes were placed in a water bath at 50°C for 2 h with shaking. The solutions were filtered through two layers of cheesecloth. The filtrate was considered the soluble protein solution. The protein concentration of the solution was then determined using AOAC method (1990) 981.10. Enzyme concentration was plotted against the percent of soluble protein in the extracted solution to determine the enzyme concentration that extracted the most protein.

Extraction time was determined using 3 g cooked chicken, 27 mL extraction buffer and an enzyme concentration equal to 0.5% (w/w) of the total protein. Centrifuge tubes were placed in the water bath at 50°C and removed at 0.25, 0.5, 1, 2 or 4 h. After filtering through cheesecloth, the soluble protein solution concentration was again determined using AOAC method (1990) 981.10. Extraction time was plotted against the

percent protein concentration to determine which extraction time extracted the most protein.

4.3.11. Cooked Storage Study (Preliminary Experiments)

Boneless, skinless chicken thighs and breasts were ground twice with a Hobart Kitchen-Aid food grinder using a 4 mm plate. Salt (1% of total meat weight) was dissolved in 10% added water and mixed in with the ground meat prior to grinding a second time. Three replicate batches of ground thigh meat and three batches of ground breast meat, were cooked in a circulating water bath as described in the previous section. Prior to cooking, a raw sample was removed and tested for hexanal. The zero time sample was removed immediately after cooking and tested prior to storage at 4°C. The remaining cooked meat was placed in resealable storage bags and stored at 4°C. Each replicate was tested for hexanal by IC-ELISA and GC at 12 h intervals for the thigh and 24 h intervals for the breast.

The samples were extracted for determination by IC-ELISA by placing 3 g cooked meat in a 50 mL test tube with 26 mL of extraction buffer (0.6M NaCl, 50 mM sodium borate; pH 9.0), 1 mL of 60 mM $NaCNBH_3$ diluted in 0.1 N NaOH and 24 μL of enzyme solution (prepared by diluting 25 μL of the enzyme in 50 mL of the extraction buffer). The tube was placed in a shaking water bath at 50°C for 3 h. After extraction, the solution was filtered through 2 layers of cheesecloth and diluted 1:10 with PBS (pH 7.2) prior to being tested in the ELISA. Protein concentration used in the ELISA, described above,

was about 2 mg protein/mL. Hexanal concentration was expressed as micrograms of hexanal per gram of meat.

4.3.12. TBA-RS Assay

Thiobarbituric acid-reactive substances of the cooked meat during storage were determined by the method of Buege and Aust (1978). After the protein was extracted from the cooked chicken and filtered through cheesecloth, 2 mL of the extract was transferred to a 15 mL centrifuge tube and 4 mL of the TBA reagent was added. The TBA reagent was comprised of 15% w/v TBA, 0.375% w/v trichloroacetic acid and 0.25N hydrochloric acid. The samples were heated in boiling water for 15 min then centrifuged at 1000 x g for 15 min. The absorbance of the supernatant was read at 535 nm and the results expressed as milligram of malonaldehyde per kilogram of meat using a molar extinction coefficient of 1.56×10^{-5} L x $mole^{-1}$ x cm^{-1}. Each sample was tested in triplicate.

4.4. Results and Discussion

4.4.1. Differential Modification of CSA

The binding inhibition produced by the differentially modified hex-CSA conjugates, when tested in the ELISA at the three dilutions, were graphed against the concentration of hexanal added to CSA (Figure 4.1). The range of binding inhibition of the conjugates at the 1/2000 dilution (5 μg CSA/mL) was the largest, ranging from 1% to 70%. The range of inhibition for the 1/1000 dilution (10 μg CSA/mL) was smaller,

ranging from 26% to 80%. The smaller range of binding inhibition may be a result of increased protein concentration which gives an increase in the concentration of hexanal that is added to the wells of the ELISA, resulting in increased inhibition. At the 1/500 dilution (20 μg/mL), the binding inhibition ranged from 12% to 79%. Although the protein concentration is doubled, the binding inhibition at the 1/500 dilution is similar to the 1/1000 dilution when CSA was modified with greater than 20 mg of hexanal/g CSA. This may be due to the protein concentration becoming too high and causing interference. Although the protein is denatured during modification, some cross-linking may still be occurring at higher protein concentrations and blocking the antibody from binding to the hexanal.

Diluting the extract from a meat sample to a volume that would contain approximately 10 μg protein/mL would be the recommended dilution to use in the ELISA for storage studies measuring lower quantities of hexanal (less than 25 mg hexanal/g protein). At this protein concentration, the ELISA has been shown to be more sensitive to lower concentrations of hexanal, giving a higher binding inhibition at of CSA lower concentrations conjugates. However, in studies measuring larger concentrations of hexanal, dilutions yielding 5 μg protein/mL would be better, as it gives a wider range of binding inhibition with changes in hexanal concentration. If the concentration of hexanal is expected to exceed that used for this differential modification study, the use of a lower protein concentration in the ELISA should be considered.

Similar results were found by Smith (1997) using ovalbumin as a carrier protein in place of CSA. The study showed a large increase in binding inhibition, 11.4% to

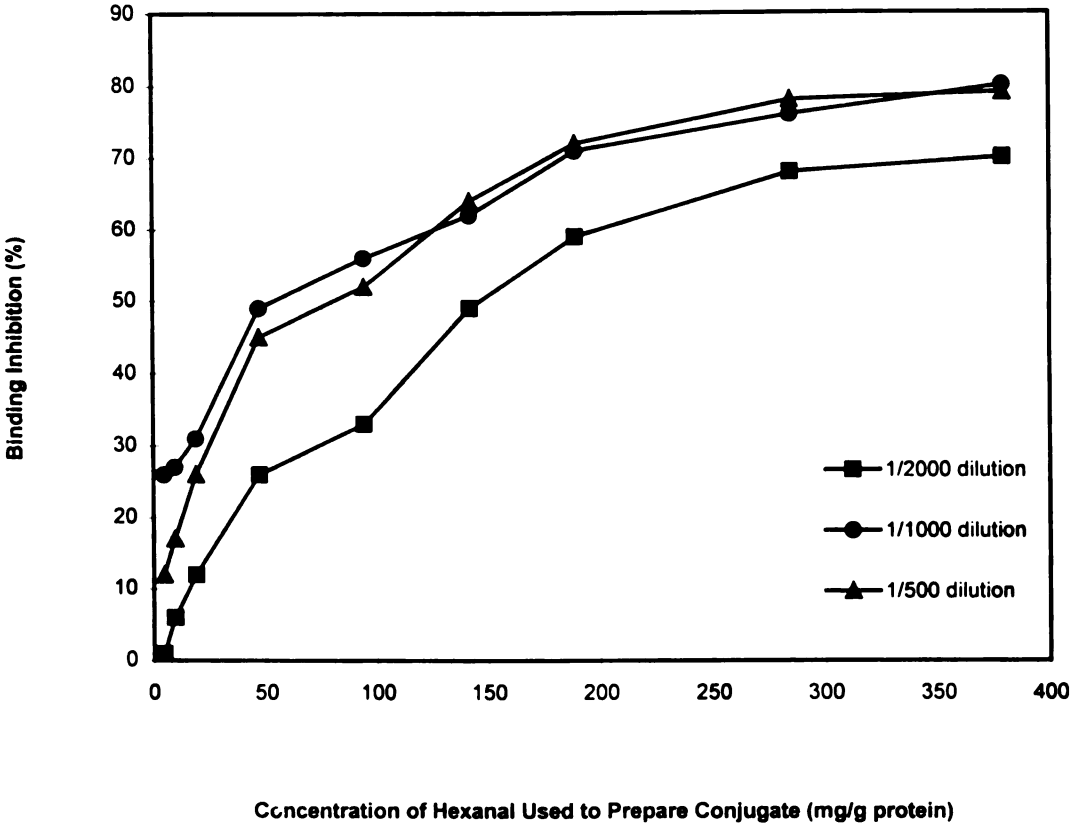

Figure 4.1. Concentration of hexanal added to prepare chicken serum albumin conjugates and the percent inhibition produced in the ELISA by three dilutions of the conjugates. Conjugates were prepared by adding 0-400 mg hexanal/g protein, then diluted 1/500, 1/1000 and 1/2000 prior to being added to the ELISA as a competitor. The calculated protein concentration of each dilution was 5 μg/mL (1/2000 dilution), 10 μg/mL (1/1000 dilution) and 20 μg/mL (1/500 dilution). Each point represents three replicates.

91.7%, as the concentration of added hexanal was increased by 25 fold. The protein concentration used in that study was 20 μg ovalbumin/mL.

The hexanal concentration of the differentially modified CSA conjugates was measured by IC-ELISA and compared to the concentration measured by GC. Hexanal concentration, measured by GC, ranged from 13.61 to 89.23 mg hexanal/g protein when 4.74 to 379.1 mg hexanal/g CSA was used for the modification. The hexanal concentration, measured by the ELISA, was varied, depending on the dilution of the conjugate (Figure 4.2.) The concentration ranged from 0.15 to 30.18 mg/g at the 1/2000 dilution. A 1/1000 dilution gave a similar range of 0.59 to 26.1 mg/g. At the lowest dilution, the concentration ranged from 0.08 to 11.69 mg/g. Although the hexanal concentration measured by GC was much higher than that by ELISA, statistical analysis of the results showed good correlation between the hexanal concentration measured by GC and the ELISA. A correlation of 0.98, 0.98 and 0.95 were calculated for the 1/2000, 1/1000 and 1/500 dilutions of the conjugates, respectively.

4.4.2 Electrophoresis and Western Blot of Salt Soluble Proteins

The hexanal modified salt soluble proteins (SSP) did not migrate as far through the gel as the corresponding unmodified proteins, suggesting the modified proteins had higher molecular weights than the native ones due to the addition of hexanal (Figure 4.3.). The antibody showed recognition of the modified chicken SSP, indicated by the presence of bands on the western blot, but showed no recognition of the native chicken proteins. It should also be noted that hexanal was conjugated to several chicken proteins.

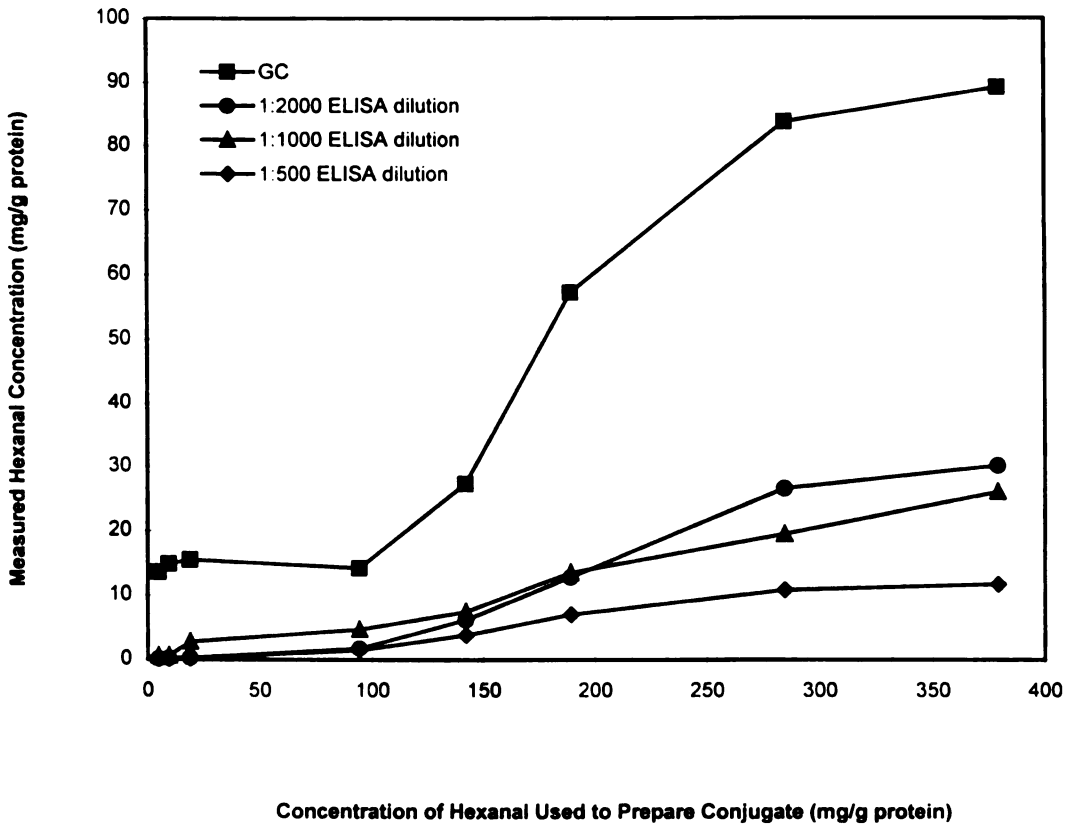

Figure 4.2. Hexanal concentration of differentially modified protein conjugates as measured by ELISA and gas chromatography (GC). The conjugates were diluted 1/500, 1/1000 and 1/2000 prior to being added to the ELISA as a competitor. The calculated protein concentration of each dilution is 5 μg/mL (1/2000 dilution), 10 μg/mL (1/1000 dilution) and 20 μg/mL (1/500 dilution). Each point represents 3 replicates.

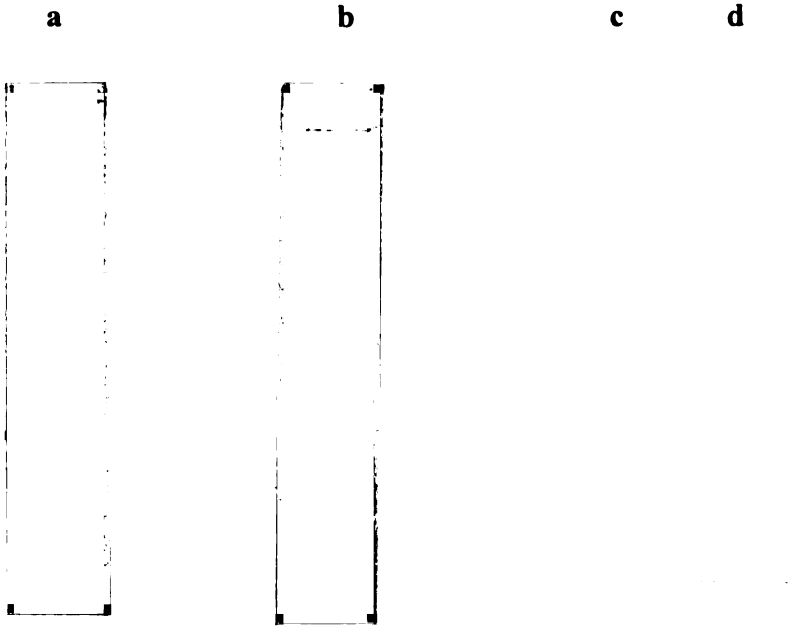

Figure 4.3. Detection of hexanal modified and native chicken breast salt soluble proteins by western blotting following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gel was stained with Commassie Blue. The western blot was performed using monoclonal antibodies against hexanal at a 1/1000 dilution. a: SDS-PAGE of hexanal modified salt soluble proteins, b: SDS-PAGE of native proteins, c: western blot of hexanal modified salt soluble proteins and d: western blot of native proteins.

4.4.3. Frozen Storage Study

Chicken thigh used in this study contained 73.3 ±0.69 % moisture, 5.29±0.1 % fat and 17.4±1.21 % protein, whereas the breast contained 74.3±5.25 % moisture, 0.93±0.07 % fat and 21.6±1.3 % protein. The hexanal concentration of the thigh, as measured by IC-ELISA, increased rapidly compared to that measured by GC (Figure 4.4). The concentration measured by the ELISA peaked at 4.02 μg hexanal/ g meat after 3 months storage. At 9 months, the hexanal concentration by GC was 2.05 μg hexanal/g meat. Although the hexanal concentration of the breast by ELISA had increased to 3.3 μg hexanal/g meat after 3 months storage, the hexanal concentration by GC had only increased to 0.42 μg hexanal /g meat after 9 months storage (Appendix A).

The difference between the hexanal concentration measured by IC-ELISA and GC may be due to changes in free and bound hexanal within the meat throughout the study. Hexanal will readily bind to protein (Gardner, 1979; Gutheil and Bailey, 1992). Since the ELISA only detects hexanal once it is bound to protein, the ELISA might initially give higher values for hexanal than GC as protein binding sites for hexanal should be readily available. Although the meat extract used in GC is heated to remove the volatiles, this temperature will remove some bound volatiles, but not all of them (Reineccius, 1996). As the oxidation reaction progresses, additional hexanal is produced, thereby resulting in more free hexanal as the protein binding sites are saturated. Since GC measures free hexanal, this would result in an increased hexanal concentration at longer storage times. Other aldehdyes will also bind to protein. Malonaldehdye has been shown to bind to

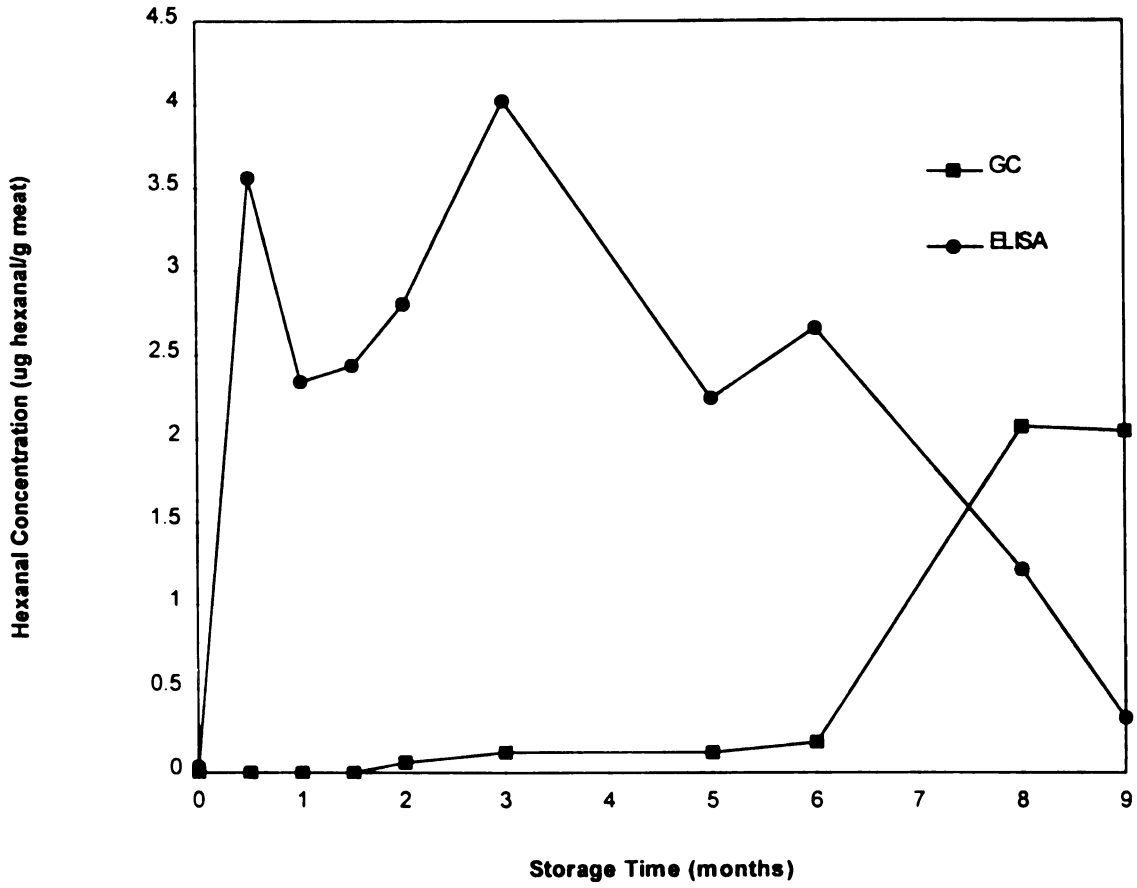

Figure 4.4. The hexanal concentration of raw chicken thigh stored at -20°C was determined by indirect competitive ELISA and gas chromatography (GC).

lysine (Crawford et al. 1967; Buttkus, 1967). It is possible that additional aldehdyes, such as malonaldehyde, are being produced over time and are displacing the hexanal, resulting in increased free and increased GC values. The ELISA values would decrease due to lack of available binding sites on the protein.

The results of another study also showed low lipid oxidation in frozen, raw chicken. Rhee et al. (1996) measured TBA numbers for raw chicken thigh and breast stored at -20°C for up to 120 days (just over 5 months). The TBA numbers for both the breast and thigh increased a small amount, 0.1 mg MA/kg meat, indicating very little lipid oxidation. They hypothesized that, although chicken muscle is high in unsaturated fatty acids, the low amount of oxidation in the breast may be due to a low concentration of metmyoglobin, an initiator of oxidation when activated by H_2O_2. The low oxidation of the thigh may be due to a high level of catalase activity, which limits H_2O_2 production, even though the thigh is more pigmented than the breast and contains more metmyoglobin.

The study by Rhee et al. (1996) also showed the oxidation of raw, frozen pork and beef. The TBA numbers for pork longissimus dorsi increased, from 0.05 to 1 mg MA/kg sample, after 75 days storage at 4°C. The TBA numbers for beef longissimus dorsi also increased, from 0.6 to 1.4 mg MA/kg sample, after 150 days storage at 4°C. Either pork or beef might be better meats to use for another frozen storage study to determine if the hexanal concentration measured by ELISA and GC can be correlated.

4.4.4. Enzyme Conditions for Cooked Meat Extraction

The concentration of alkaline protease and extraction time needed to maximize protein solubility of the cooked chicken proteins was determined. The soluble protein concentration of the cooked meat extract was 0.88% without enzyme and increased to 1.69% when enzyme concentration was increased to 0.5% of the total protein concentration and incubated at 50° C for 2 h (Table 2.1.). Total protein concentration of chicken thigh is 20.08% (USDA, 1978). The meat is diluted 1/10 during the extraction process, giving a theoretical maximum protein concentration of 2%. Soluble protein concentration increased only slightly (a 0.12% increase) when enzyme concentration was increased to 1% of total protein. Although the enzyme is further diluted (1/50) prior to ELISA analysis, thereby decreasing the concentration, and would have been at a pH and temperature outside of its optimal range (Enzyme Development Corporation information sheet), the 0.5% enzyme concentration was used to be certain there would be no proteolysis of the antibody by the enzyme.

Using a 0.5% (wt/vol) concentration of the enzyme, the effect of extraction time at 50°C on soluble protein concentration was determined. The soluble protein concentration increased from 0.86 to 1.99% during the 0.25 to 4 h incubation (Table 2.2.). A 3 h extraction time was chosen so that the ELISA could be completed within a period of time comparable to the GC procedure. The final extraction conditions were set at 50°C for 3 h using an enzyme concentration of 0.5% of the total protein.

Table 4.1. Effect of enzyme concentration on the extraction of salt soluble protein from cooked chicken thigh at 50°C for 2 h.

Enzyme Concentration (%)[1]	Soluble Protein (%)[2]
0	0.88 ± 0.09
0.05	1.02 ± 0.03
0.1	1.23 ± 0.05
0.2	1.36 ± 0.18
0.5	1.64 ± 0.11
1.0	1.79 ± 0.04

[1] Enzyme concentration was expressed as % of total protein in chicken thigh.
[2] Theoretical maximum protein concentration of the extract was 2%.

Table 4.2. Effect of extraction time on the salt soluble protein concentration of cooked chicken thigh at 0.5% enzyme concentration and 50°C.

Time (h)	Soluble Protein (%)[1]
0.25	0.86 ± 0.05
0.5	1.14 ± 0.04
1.0	1.42 ± 0.13
1.5	1.55 ± 0.06
2.0	1.68 ± 0.13
4.0	1.99 ± 0.13

[1] Theoretical maximum protein concentration of the extract is 2%.
Enzyme concentration was expressed as % of total in chicken thigh.

4.4.5. Cooked Storage Study

Prior to cooking, the chicken breast in this study contained 70.7± 1.67% moisture, 1.34± .03% fat and 21.2± 0.35% protein. Although the breast meat showed an increase in hexanal concentration during storage when measured by GC, it did not show an increase by ELISA. The hexanal concentrations obtained for the breast by GC and ELISA, as well as the TBARS numbers are shown in Appendix A.

Chicken thigh in this study contained 73.3±0.69% moisture, 5.29± 0.1% fat and 16.8± 1.21% protein. The hexanal concentration of the thigh, measured by GC, increased rapidly from 2.48 to 12.41 μg hexanal/g meat over 60 h of storage. The hexanal values by GC for the thigh in this study are comparable to the values reported by other researchers. Dupuy et al. (1987) reported the hexanal concentration of dark meat from roast turkey to be 11.7 μg hexanal/g meat after 2 days storage at 4°C. The hexanal concentration of uncured, cooked chicken after 24 h storage at 4°C was 9.84 μg hexanal/g meat (Ramarathnam et al., 1991).

The TBARS value also showed an increase similar to the GC results, from 1.17 to 13.01 mg MA/kg meat after 60 h storage at 4°C. These values are also comparable to values reported by other researchers. The TBA number of cooked chicken thigh stored for 6 days at 4°C was 14.5 mg MA/kg sample (Rhee et al., 1996). The TBA number of raw chicken thigh increased by a factor of 10 after 8 days storage at 4°C (Whang and Peng, 1987).

The hexanal concentration measured by ELISA increased from 0.71 μg hexanal/g meat to 1.41 μg hexanal/g meat after 12 h of storage (Figure 4.7.). Hexanal concentration

by ELISA for the cooked chicken may be lower than GC due to the loss of free lysine sidechains due to binding with other aldehydes and ketones formed during storage. The TBARS numbers of the cooked thigh increased rapidly, a possible indication that malonaldehdye was being formed. Crawford et al. (1967) found the ε amino group of lysine in bovine serum albumin was blocked after exposure to MA. Buttkus (1967) found that, after exposure to MA, ninhydrin reactive amino groups in trout myosin were lost. Ninhydrin will react only with free amino groups, especially the ε amino of lysine. Another study noted that as 2-thiobarbituric acid (TBA) values increased, free ε amino groups decreased (Kuusi et al., 1975). It is possible that malonaldehdye is binding to the lysine side chains.

A possible solution to the problem of lysine not being available for binding to hexanal would be to add in a pure protein, such as CSA, during extraction. Free hexanal could then bind to the CSA and the hexanal concentration could be determined based on the amount of CSA added. This could then be converted to the hexanal concentration in the meat. Another possible source of lysine would be to add ε-amino caproic acid. Since the antibody did not cross-react 100% with ε-amino caproic acid, the lower amount of cross reactivity could be adjusted for when calculating the hexanal concentration.

Another possible reason for the lower hexanal concentration measured by the ELISA may be not enough sodium cyanoborohydride to sufficiently reduce the double bonds formed between hexanal and the meat proteins. Additional studies will need to be conducted to determine if the concentration of sodium borohydride needs to be increased.

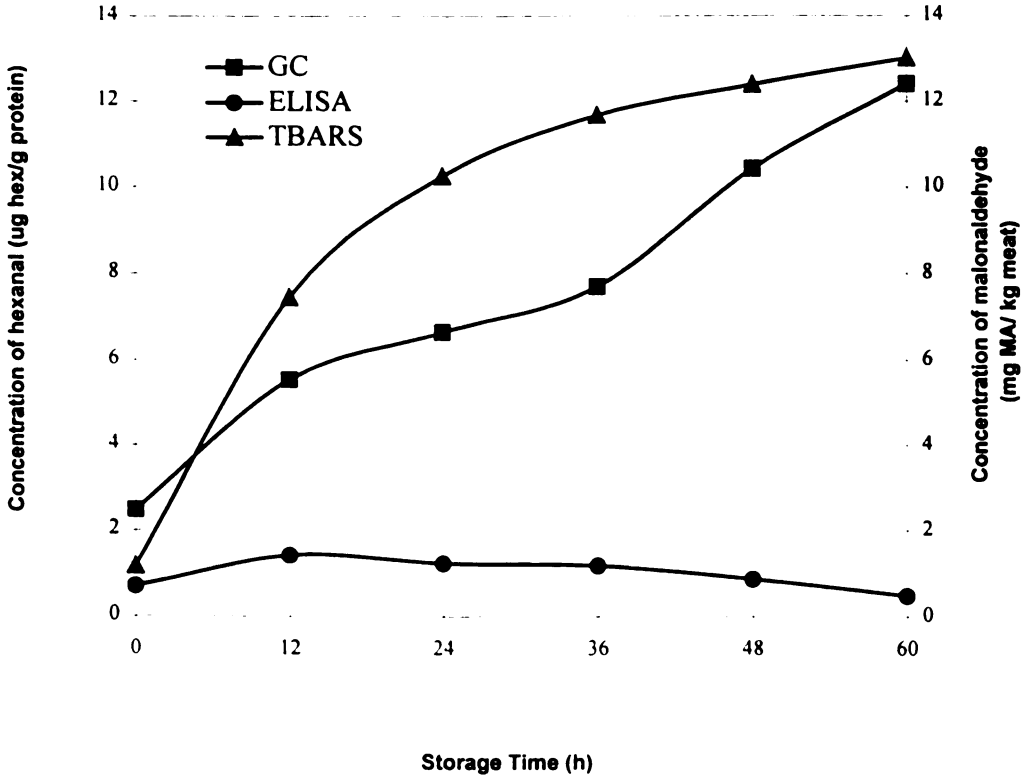

Figure 4.5. Hexanal concentration and 2-thiobarbituric acid reactive substances (TBARS) number of cooked chicken thigh meat stored at 4°C. Hexanal concentration was determined by both ELISA and gas chromatography.

The results of the study with the differentially modified CSA suggest that the ELISA could be used in place of the GC method. The correlation between GC and all three dilutions of the conjugates was high. However, when the ELISA was used in actual meat storage studies, the results were not conclusive. No correlation was found between the two methods. Further studies are required to determine if the ELISA can be used as a reliable indicator of lipid oxidation in meat systems.

Chapter 5

CONCLUSIONS

1. We produced highly sensitive monoclonal antibodies to hexanal modified bovine serum albumin. Sodium cyanoborohydride was used as the reducing agent, allowing us to reduce the Schiff bases formed between hexanal and lysine. The antibody was found to recognize the hexanal-lysine complex.

2. We found three compounds, in addition to hexanal, that the antibody cross-reacted with. The compounds were pentanal, 2-*t*-hexenal and heptanal, aliphatic aldehydes with 5, 6 and 7 carbons. Although the monoclonal antibody cross-reacted with these three compounds, the assay is still useful for monitoring lipid oxidation, as both pentanal and heptanal are lipid oxidation products that contribute to oxidized flavors. also, the concentrations of these three compounds that have been measured in foods are insignificant compared to the levels of hexanal commonly found.

3. We determined that the monoclonal antibody ELISA correlated well with a commonly used GC method when using differentially modified chicken serum albumin conjugates.

Chapter 6

FUTURE RESEARCH

Although the ELISA showed promise by correlating with GC using the differentially modified conjugates, the results of the storage study were inconclusive. More work needs to be done to better understand the binding of hexanal to chicken proteins and the measurement of hexanal concentrations by ELISA.

A frozen storage study may be more useful than a refrigerated study as oxidation occurs slower. By using a meat product, such as beef or pork, that oxidizes quickly at frozen temperatures, the ELISA method could be perfected. After a better understanding of the ELISA is determined, a refrigerated study could be undertaken. Further research is needed to determine the availability of lysine during oxidation of chicken meat at 4° C. The possibility of adding in a set concentration of protein needs to be examined as a source of lysine. Once the ELISA method has been verified by GC for an actual meat system, the ELISA could then be verified through sensory analysis.

In addition to detecting hexanal concentration in meats, the ELISA could be examined for use in detection of hexanal concentration in non-meat products. Protein would need to be added as a carrier if the product has a low protein concentration. If additional protein is added for more lysine in the meat system, it could then be easily used in another product.

APPENDIX A

APPENDIX A

Conversion Example

An example of the conversion of 1 μg protein/mL concentration of hex-CSA to hexanal concentration is shown here.

$$\frac{1\ \mu g\ protein}{mL} \times \frac{46\ moles\ lysine}{1\ mole\ protein} \times \frac{1\ mole\ protein}{66{,}300\ gram} \times \frac{1\ gram}{10^6\ \mu g} \times \frac{10^6\ moles}{1\ mole} = \frac{7.4 \times 10^{-4}\ \mu moles\ lysine}{mL}$$

The lysine concentration is multiplied by 99.1% to account for the % modification. Since one mole of lysine will bind one mole of hexanal, the moles are considered equivalent. Therefore, there are 7.3×10^{-4} μmoles of hexanal.

$$\frac{7.3 \times 10^{-4}\ \mu mole\ hexanal}{mL} \times \frac{100\ gram}{1\ mole} \times \frac{1\ mole}{10^6\ \mu\ mole} \times \frac{10^9\ ng}{1\ gram} = \frac{73\ ng\ hexanal}{mL}$$

APPENDIX B

APPENDIX B

Values Obtained for Oxidation of Breast

Frozen Study Results

	Hexanal Concentration (μg /g meat)	
Month	GC	ELISA
0	0	0.018
0.5	0	4.15
1	0	2.13
1.5	0	2.62
2	0	2.38
3	0	3.30
5	0	1.96
6	0	2.20
8	0.51	1.81
9	0.42	0.37

Cooked Study

	Hexanal Concentration (μg/mL)		
Day	GC	ELISA	TBARS (mg MA/kg sample)
0	1.92	0.43	3.31
1	5.25	0.46	5.48
2	6.01	0.24	6.62
3	7.06	0.03	6.89
4	5.84	0.64	8.27
5	4.1	0.29	7.55
7	1.82	0.07	7.79

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