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PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES TO HEXANAL-LYSINE ADDUCTS FOR USE IN AN ELISA TO MONITOR LIPID OXIDATION IN A MEAT MODEL SYSTEM

By

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ABSTRACT

PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES TO HEXANAL-LYSINE ADDUCTS FOR USE IN AN ELISA TO MONITOR LIPID OXIDATION IN A MEAT MODEL SYSTEM

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Hexanal content is a popular index of lipid oxidation in foods, including meats. Lipid oxidation in meat is generally associated with development of objectionable flavors potentially limiting shelf-life and causing loss of consumer acceptability. Interest in new methods for measuring the extent of lipid oxidation has arisen due to limitations of standard approaches, such as thiobarbituric acid (TBA) assay and headspace gas chromatography (HS-GC). While TBA assays are sensitive and simple, they are non-specific. While HS-GC methods are sensitive and specific, they are typically time-consuming and often difficult. Enzyme-linked immunosorbent assays (ELISAs) are considered sensitive, specific, rapid and easy-to-use. The goal of this study was to develop an ELISA incorporating hexanal-specific antibodies to measure hexanal in meat.

We developed and characterized highly sensitive and specific polyclonal antibodies against hexanal-modified bovine serum albumin. The resultant antibodies recognized hexanal conjugates, but not free hexanal in 0, 10, 20 or 30% methanol. Reactivity occurred with hexanal conjugates containing a protein as large as keyhole limpet

hemocyanin (400 kD) or a compound as small as ε-aminocaproic acid, a lysine derivative (131 D). In contrast, the antibodies reacted much more specifically with the "hapten-end" of conjugates. When chicken serum albumin was modified with other aldehydes, ketones, and alcohols, instead of hexanal, only two compounds showed substantial cross-reactivity. Heptanal, the aliphatic aldehyde with one more carbon than hexanal, and pentanal, with one less carbon than hexanal, had cross-reactivities of 86.3% and 11.8%, respectively. This polyclonal-based ELISA had a detection limit of 1.5 nanogram hexanal per milliliter solution with a mean interplate coefficient of variation of 9.3% (n = 281). The assay was used to assess the extent of lipid oxidation resulting from incubation of chicken thigh muscle homogenate at 50°C. Comparison of these results with results obtained by a TBA assay and a dynamic HS-GC method showed high correlations (r = 0.85 and r = 0.89, respectively). A monoclonal antibody to hexanal-lysine adducts, produced in a separate study, was also incorporated into an ELISA and used to measure hexanal content. These results correlated similarly with standard methods. Further study of lipid oxidation in muscle foods using immunoassays is recommended.

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CHAPTER 1

INTRODUCTION

The poultry industry has grown tremendously especially with the development of further-processed chicken products (Kinsman, 1994). A dramatic increase in the amount of chicken consumed domestically has occurred since 1970 (Kinsman, 1994). In addition, U.S. broiler meat exports hit a record-high of $1.7 billion in 1995 and forecasts are $2.5 billion for 1997 (Thornton, 1996). Consumer acceptance of meat products depends to a major extent on flavor quality (Ramarathnam et al., 1991a). Poultry muscle is very susceptible to oxidative deterioration resulting in off-flavor development because of its relatively high concentration of unsaturated fatty acids and low concentration of tocopherols (Ladikos and Lougouvois, 1990). Unfortunately, the potential for lipid oxidation and rancid or warmed-over flavor development is one of the single greatest constraints in determining the processing and shelf-life characteristics of muscle foods (Allen and Foegeding, 1981).

A uniform and satisfactory method to evaluate flavor quality and stability of lipid foods needs to be developed (Min, 1981). Fifteen years later, the subject of appropriate measurements continues to be one of considerable debate among researchers. In May 1996, at the American Oil Chemists' Society (AOCS) Annual Meeting and Expo in Indianapolis, the AOCS Antioxidant Common Interest Group sponsored a panel

discussion on the topic (Cuppett and Warner, 1996). The discussion was organized to initiate a dialog on accepted protocols for antioxidant research in foods and biological systems and the panel was composed of a diverse group of international researchers. While no consensus was reached regarding available protocols, several key points were made. First, the diversity of methodologies currently in use to evaluate the progress of oxidation in both food and biological systems has created difficulty in interpreting and comparing the published data of different researchers. Second, prior to selecting methods, researchers should clearly define: 1) the study objectives, such as kinetics, reaction mechanisms, and antioxidant effectiveness; 2) the oxidation system, such as bulk oils, emulsions, or biological materials; 3) the oxidation targets, such as lipids or proteins; and 4) the oxidation endpoints, such as induction periods, tissue damage or quality changes. Third, no progress will be made if researchers continue to use archaic tests that measure nonspecific products in complex food and biological systems.

For purposes of food analysis, it is extremely doubtful that a reliable instrumental method will replace the human being for sensory evaluation of foods in the near future (Reineccius, 1996). While the most commonly used technique for flavor analysis is gas chromatography, its use for non-research applications, such as quality assurance, has been limited due to time constraints and method complexity (Reineccius, 1996). Immunoassays offer a rapid, cost-effective, and easy-to-use alternative, achieving sensitivity and specificity without requiring highly trained analysts or sophisticated equipment (Samarajeewa et al., 1991). As an analytical tool, the immunoassay has already proven to be very useful for the assessment of a variety of substances in meat products (Fukal, 1991).

To assess off-flavor development due to lipid oxidation in foods before an unacceptable level is reached, a novel analytical approach which is both sensitive and specific is needed. For testing to be performed easily in a processing or storage facility, the assay should be simple and rapid as well. The overall goal of this research was to develop an enzyme-linked immunosorbent assay (ELISA) to assess the extent of lipid oxidation in muscle foods. The criteria set forth by Gray (1978; Gray and Monahan, 1992) were used to evaluate our rationale. The criteria are as follows:

- Specificity of property to be measured (hexanal content) for lipid oxidation.
- Degree to which property represents extent of lipid oxidation.
- Specificity of method (ELISA) for property.

First, hexanal is a secondary product of lipid oxidation. Hexanal is produced during autoxidation of all compounds with a double bond in the ϖ-6 position, including 9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD), 13-HPOD, 2,4-decadienal, and 2-octenal (Schieberle and Grosch, 1981). In meats, oleic, linoleic and arachidonic acids are the primary reactants of lipid oxidation (Ladikos and Lougouvois, 1990) with the latter two fatty acids degrading to hexanal. Hexanal is also a major product of lipoxygenase-hydroperoxide lyase activity in a variety of plant tissues (Hsieh, 1994).

Second, hexanal is one of the compounds contributing to the undesirable odor and flavor associated with rancidity (Pearson et al., 1977). Due to its low odor threshold and relatively high concentration in oxidizing foods, hexanal has flavor significance as well as indicating lipid degradation. Over thirty years ago, hexanal was recognized as a useful indicator of oxidative flavor deterioration in stored foods, specifically in potato granules (Boggs et al., 1963), uncured, canned ham (Cross and Ziegler, 1965) and fried chicken

(Nonaka and Pippen, 1966). Hexanal has long been used as an index of oxidative deterioration in potato products (Salinas et al., 1994) and is very commonly used to monitor hexanal content in vegetable oil due to its well-established correlation with oxidation in that system (Reineccius, 1996). Hexanal appears to be a sensitive and reliable indicator for assessment of the oxidative condition and flavor quality of meat and meat products (Shahidi, 1994). For example, strong correlations have been reported between sensory scores and hexanal content for cooked ground beef (St. Angelo et al., 1987) and ground pork (Shahidi et al., 1987) with coefficients of 0.80 and 0.98, respectively.

Third, immunoassays are based on the inherently highly specific immunological interaction between an antibody and a corresponding antigen (Hefle, 1995). Therefore, we expect that the use of antibodies against hexanal in an ELISA will yield a detection system highly specific to hexanal.

The first objective of this study (Chapter 3) was to produce and characterize sensitive polyclonal antibodies specific to hexanal to be incorporated into an ELISA. The second objective (Chapter 4) was to compare the ELISA to a "standard" gas chromatography method for hexanal to monitor lipid oxidation in a model system. A review of the literature (Chapter 2) further supports our selection of hexanal as an indicator compound and immunoassay as our analytical technique. The sensitivity and cross-reactivities of our anti-hexanal polyclonal serum were assessed and are discussed in Chapter 3. Application of two competitive indirect ELISAs, one using our polyclonal antiserum and one using a hexanal-specific monoclonal antibody, to measure lipid oxidation in a chicken muscle model system is shown and discussed in Chapter 4.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipid Oxidation Mechanisms

Oxidative deterioration of unsaturated lipids results in the formation of volatile and nonvolatile secondary breakdown products via hydroperoxide formation. Hydroperoxide formation occurs nonenzymatically by one of two mechanisms, autoxidation or photosensitized oxidation. Autoxidation begins with hydrogen abstraction from allylic methylenes, in the presence of trace metals, light or heat, producing allylic radicals which react with molecular oxygen producing peroxy radicals. Subsequent hydrogen abstraction from another unsaturated fatty acid by the peroxy radical results in the formation of a hydroperoxide. Photoxidation begins as a direct addition of oxygen, activated to the singlet state by exposure to light and a sensitizer, to the carbon-carbon double bond yielding hydroperoxides formed at either unsaturated carbon. Each mechanism yields different isomeric distributions of hydroperoxides from the same fatty acid (Frankel, 1984) and subsequently forms different types and amounts of secondary products.

Hydroperoxide decomposition involves a very complicated set of reaction pathways (Frankel, 1984) yielding a complex mixture of volatile and nonvolatile secondary and tertiary reaction products, including acids, alcohols, aldehydes, esters, furans, hydrocarbons, ketones, and lactones. Monohydroperoxides are very unstable and readily

decompose through homolytic cleavage of the hydroperoxide group to an alkoxy radical and a hydroxy radical. The alkoxy radical undergoes β-scission of the carbon-carbon bond via one of two routes. Scission route a is a cleavage of the carbon-carbon bond on the side of the oxygen-bearing carbon further from the double bond forming an unsaturated aldehyde and an alkyl radical which can react with a hydroxyl radical to form an alcohol. Scission route b is a cleavage of the carbon-carbon bond between the double bond and the oxygen-bearing carbon forming a saturated aldehyde and vinyl radical which can react with a hydroxyl radical to form a 1-enol which tautomerizes to the corresponding aldehyde. Alternative pathways of hydroperoxide decomposition are further oxidation, yielding epoxides, cyclic and bicyclic peroxides, and condensation, yielding dimers and polymers which subsequently may oxidize and decompose into volatile products (Ladikos and Lougovois, 1990). Malonaldehyde, another common lipid oxidation product, arises from cyclic endoperoxides produced by autoxidation of fatty acids containing three or more double bonds. Other tertiary reactions, including decomposition of non-volatile secondary products and further oxidation of unsaturated aldehydes and ketones, contribute to the complexity of the flavor profile of an oxidized food (Kochhar, 1993).

The aliphatic aldehydes are the most important volatile breakdown products because they are the major contributors of unpleasant odors and flavors in food products (Kochhar, 1993). Each unsaturated fatty acid gives a characteristic pattern of aldehydes via oxidation (Esterbauer, 1982). Although the specific production pathways of all the aldehydes found in various oxidized samples have not been elucidated, most of the aldehydes are formed by one or more of the chain scission reactions (Esterbauer, 1982).

Oleic acid may generate octanal, nonanal or decanal while linolenic and arachidonic acids may generate propanal and hexanal, respectively (Table 2.1).

Among the most important precursors of aldehyde compounds is linoleic acid due to its abundance in foods and high susceptibility to oxidation (Kochhar, 1993). Autoxidation of linoleic acid produces hexanal via 13-hydroperoxyoctadeca-9,11-dienoic acid hydroperoxide following homolytic clevage scission route b (Figure 2.1) and 2,4-decadienal via 9-oxo-nonanoic acid hydroperoxide following route a (Figure 2.2). Further oxidation of 2,4-decadienal generates hexanal and 2-octenal (Schieberle and Grosch, 1981), while subsequent oxidation of 2-octenal yields heptanal and hexanal. In fact, all compounds containing an ω-6 double bond produce hexanal (Schieberle and Grosch, 1981).

Experimental determination of the volatile carbonyl compounds arising from autoxidation of linoleic acid at low and moderate temperatures indicates that hexanal is the major component comprising 66 mol %, while 2-octenal, 2-heptenal, and 2,4-decadienal are minor components comprising 18, 6, and 5 mol %, respectively (Badings, 1970). The fact that hexanal is the only aldehyde to arise from both linoleic acid hydroperoxides, as well as from some of the aldehydic breakdown products, explains its predominance as a linoleic acid autoxidation product (Schieberle and Grosch, 1981). In addition, hexanal is protected against further oxidation to some extent by the more reactive unsaturated aldehydes present in a mixture (Schieberle and Grosch, 1981), as in the case of a food system.

Lipid oxidation is a non-enzymic process in that there is no direct reaction of an enzyme with an unsaturated fatty acid (Kanner, 1994). Lipoxygenase (LOX) does,

Table 2.1 Fatty acid precursors, hydroperoxide intermediates and β-scission reaction pathways of alkoxyl radicals forming various saturated aldehydes

Fatty Acid	Hydroperoxide	Scission Route	Aldehyde
Oleic acid	8-OOH	b	Decanal
	9-OOH	b	Nonanal
	10-OOH	b	Nonanal
	11-OOH	b	Octanal
Linoleic acid	13-OOH	b	Hexanal
Linolenic acid	16-OOH	a	Propanal
Arachidonic acid	15-OOH	a	Hexanal

Adapted from Kochhar (1993)

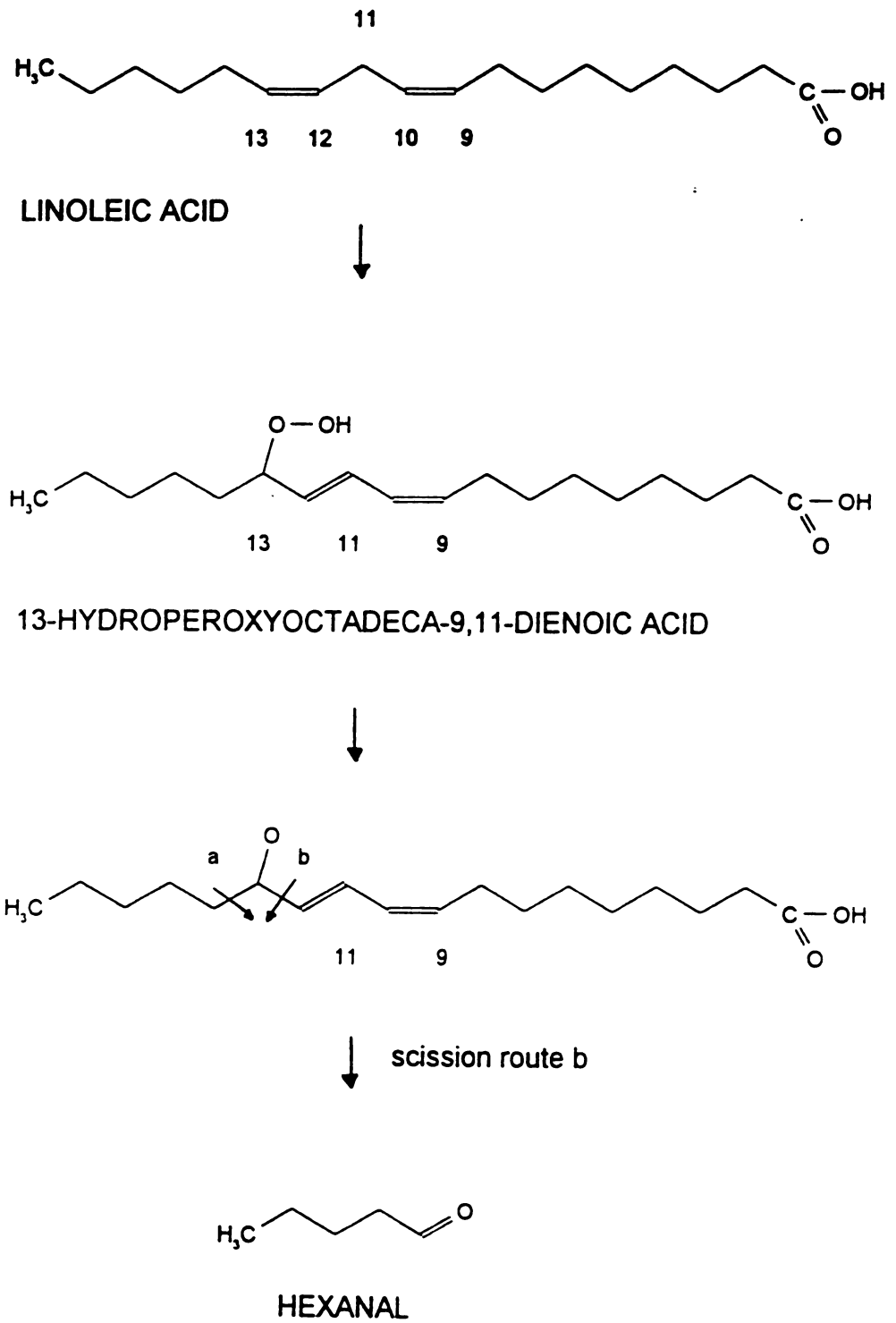


Figure 2.1. Autoxidation of linoleic acid: Breakdown of 13-hydroperoxide to hexanal via β-scission route b.

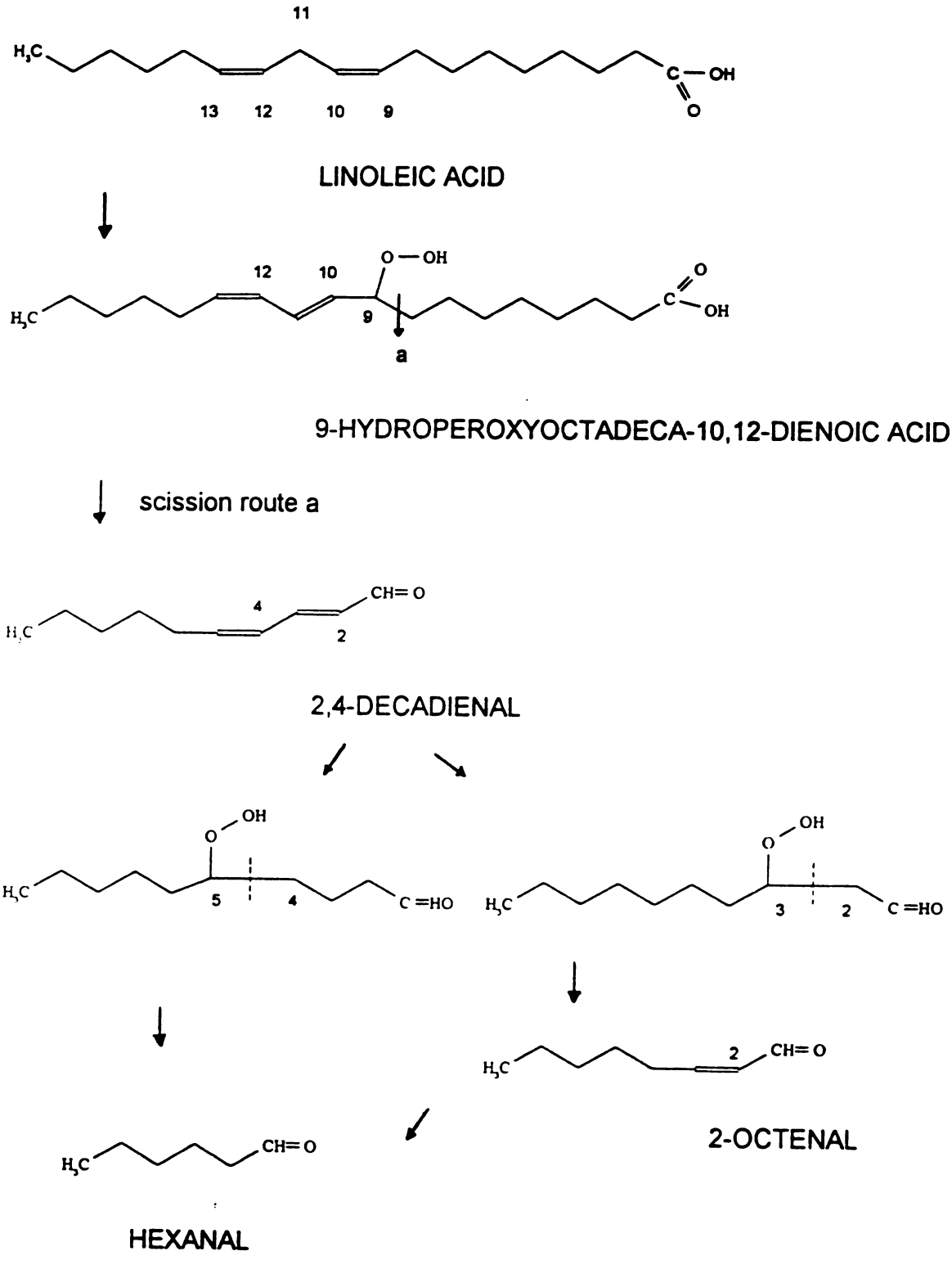


Figure 2.2. Autoxidation of linoleic acid: Breakdown of 9-hydroperoxide to hexanal via β-scission route a.

however, participate in oxidative deterioration of biological systems by catalyzing oxidation of certain fatty acids (Hsieh, 1994). Activated LOX can abstract hydrogen from polyunsaturated fatty acids containing a 1,4-cis,cis-pentadiene system (Hsieh, 1994). To become activated, LOX requires fatty acid hydroperoxides (Kanner, 1994) to oxidize its iron moiety from the ferrous to ferric state (Hsieh and Kinsella, 1989). Various LOX isozymes exist differing in the position of hydrogen abstraction and oxygen addition, the direction of double bond shift, and in relative affinities for different fatty acids (Hsieh and Kinsella, 1989). Hence, they generate different types and amounts of products. For example, linoleic acid is oxidized by lipoxygenase isozyme 1 (LOX-1) producing approximately 90% c-13 monohydroperoxide and 10% of the c-9 isomer, whereas LOX-2 and LOX-3 produce 40% and 60% of the isomers, respectively (MacLeod and Ames, 1988). A second enzyme, a hydroperoxide lyase, acts specifically on 13-hydroperoxide of linoleic acid to produce hexanal.

2.2 Factors Influencing Lipid Oxidation in Food

Lipid oxidation is considered a primary cause of quality deterioration in foods, especially in muscle tissues (Love and Pearson, 1971; Melton, 1983; Rhee, 1988; Asghar et al., 1988; Rahargo and Sofos, 1993; and Kanner, 1992). Numerous internal and external factors influence the rate of lipid oxidation in a food system. The internal factors are system components, such as fatty acids and metal ions (Pearson et al., 1977), and system characteristics, such as water activity (Fritsch and Gale, 1977). External factors include storage temperature, time, light, and oxygen availability (Hsieh and Kinsella, 1989).

The degree of unsaturation of the fatty acid constituents of the lipids is a primary factor dictating the reaction rate (Shahidi and Pegg, 1994). In general, the rate of autoxidation increases as the number of double bonds increases. In meats, linoleic, oleic, and arachidonic acids are the primary reactants during lipid oxidation (Ladikos and Lougovois, 1990). The relative oxidative susceptibility of lipids by species, based on the polyunsaturated fatty acid content, in decreasing order is fish, poultry, pork, beef, and lamb in both adipose fat (Pearson et al., 1977) and phospholipids (Kanner, 1994). Despite the low concentration of phospholipids relative to adipose fat in meat tissue, the combination of the high degree of unsaturation of their constituent fatty acids and their close proximity to lipid oxidation catalysts make phospholipids primary targets of oxidative reactions (Drumm and Spanier, 1991). Transition-metal ions can stimulate lipid oxidation by either the generation of initiators, e.g. hydroxyl radical, or the decomposition of hydroperoxides into peroxy and alkoxy radicals (Halliwell, 1995). Both free and protein-bound iron are capable of catalyzing oxidation of unsaturated fatty acids (Pearson et al., 1977; Ladikos and Lougovois, 1990). Sources of protein-bound iron existing in biological tissue include myoglobin, hemoglobin, and cytochromes. Reducing compounds are the driving forces in metal ion-catalyzed lipid oxidation (Kanner, 1994) and ascorbic acid is the main election donor for the iron-redox cycle in muscle tissue (St. Angelo, 1996). Ascorbic acid can either promote or inhibit lipid oxidation depending on its concentration. Low concentrations generally promote oxidation by causing reduction of metal ions (Ladikos and Lougovois, 1990); high concentrations inhibit oxidation by donating hydrogen and inactivating free radicals (Decker and Hultin, 1992). While the exact ascorbic acid concentration producing a given effect is dependent on the iron

concentration, at the concentrations of each found in animal tissue, ascorbic acid is most likely a prooxidant (Decker and Hultin, 1992). Sodium chloride has a significant prooxidant effect, while phosphates act as antioxidants (Gray et al., 1994). Water activity is another factor influencing lipid oxidation. For most foods, the lowest reaction rate occurs at a water activity of between 0.2 and 0.3. Water acts as an antioxidant by decreasing the catalytic activity of transition metals; below 0.1, significant acceleration occurs (Fritsch, 1994). The storage stability of dry foods such as cookies is mostly limited by lipid oxidation (Lingnert, 1980).

External factors affecting lipid oxidation during both processing and storage must be considered since they can be manipulated to control the oxidative susceptibility inherent in some food systems. In muscle tissue lipids, oxidative flavor deterioration can arise either rapidly as in refrigerated, cooked meats, apparent within 48 hours at 4°C. Oxidative flavor deterioration can also arise slowly as in raw meat and fatty tissue, usually requiring months of freezer storage (Spanier et al., 1992). It is now generally accepted that the former process, producing a condition referred to as warmed-over flavor (WOF), is not limited to cooked meat. Rapid off-flavor development is promoted in raw meat by any process involving disruption of the muscle structure in the presence of air, including cooking, grinding, or restructuring (St. Angelo, 1996) causing release of free iron and catalyzing oxidation (Drumm and Spanier, 1991). While cooked meat is more susceptible to lipid oxidation than raw meat, when raw meat is subjected to size reduction, temperature abuse and/or prolonged storage, oxidation can become a serious problem (Rhee, 1988). Disruption of tissue is an important mechanism for the formation of aldehydes, ketones, alcohols, and oxoacids in many plant as well as animal tissues (Hsieh

and Kinsella, 1989), since increased oxygen availability accelerates oxidation of unsaturated fatty acids (Muller and Gautier, 1994). In low moisture foods, the drying process creates channels as water is removed, allowing rapid migration of oxygen, and also ruptures some lipid globules increasing the lipid surface area (Fritsch, 1994). A low storage temperature, protection from light and oxygen, and a favorable water activity all improve the oxidative stability of a food system containing lipid.

Lipoxygenases are present in many plant, animal, poultry, and fish tissues producing flavor compounds desirable in some foods and offensive in others (Hsieh, 1994). Hexanal is a major product of the activity of a lipoxygenase-hydroperoxide lyase enzyme system in a variety of plants, including apple, banana, corn, cucumber, green bean and tomato (Hsieh, 1994; Muller and Gautier, 1994). Hexanal contributes to the acceptable, characteristic "green" or "fresh" flavor in some foods, such as in green beans (Hsieh, 1994). Hexanal also yields undesirable "green" or "beany" flavor in others, as in soy products (MacLeod and Ames, 1988). Both plant and animal lipoxygenases can use linoleic acid as a substrate; however, there is no reported direct correlation between lipoxygenase activity and flavor generation in muscle foods (Hsieh, 1994). In raw muscle foods, lipid oxidation may be catalyzed by activated lipoxygenases; while in cooked, stored meat products, lipid oxidation is completely dependent on nonenzymatic catalysis (St. Angelo, 1996).

2.3 Flavor Significance of Oxidative Products

To evaluate the organoleptic importance of a volatile compound arising from lipid oxidation, it is necessary to know its threshold value and concentration in the sample

(Frankel, 1982; Gray and Monahan, 1992). Certain compounds present in trace quantities have such an intensive odor they are significant to the overall flavor profile (Frankel, 1982). Hexanal is one of the compounds contributing to the undesirable odor and flavor associated with rancidity (Pearson et al., 1977). Characterized as having a "green" odor as a pure compound, hexanal has a threshold in oil of 80 ppb (Mottram, 1987). Hexanal and other aldehydes have been used successfully to follow lipid oxidation in meat products (Gray and Monahan, 1992). An increase in lipid-derived volatile compounds, including hexanal, closely follows changes in sensory descriptors, such as painty, used by panelists (Spanier et al., 1992).

2.4 Hexanal as an Indicator of Lipid Oxidation

Hexanal content appears to be a sensitive and reliable indicator for evaluation of the oxidative state and flavor quality of meat and meat products (Shahidi, 1994). Hexanal content has been monitored during refrigerated storage of cooked meats including: ground pork (Shahidi et al., 1987); ground beef (St. Angelo et al., 1988); beef (St. Angelo et al., 1987); roast turkey, chicken, and beef (Dupuy et al., 1987); turkey rolls (Wu and Sheldon, 1988); roast pork (Robson et al., 1989); broiler breast, thigh, and/or skin (Ang and Young, 1989; Ang and Lyon, 1990; Ajuyah et al., 1993); chicken patties (Su et al., 1991; Ang and Huang, 1993); chevon (Lamikanra and Dupuy, 1990); and restructured beef steaks (Stoick et al., 1991). The concentration of hexanal has also been monitored during frozen storage of raw meats, including ground pork (Brewer et al., 1992), restructured beef steaks (Stoick et al., 1991), and alligator meat (Cadwallader et al., 1994), and of cooked meats, including beef slices (Hwang et al., 1990), ground turkey

patties (Craig et al., 1991), and restructured chicken nuggets (Lai et al., 1995). Measurement of hexanal has been used to assess the extent of lipid oxidation in canned tuna (Przybylski et al., 1991) and to compare cured and uncured meats, including ham (Cross and Ziegler, 1965), pork (Ramarathnam et al., 1991b), and beef and chicken (Ramarathnam et al., 1991a). Larick et al. (1992) found that pork from pigs fed diets containing 4 or 6% linoleic acid had higher concentrations of hexanal than pigs fed diets of 2% linoleic acid. The authors suggest that while a more unsaturated fatty acid profile may be desirable, the impact of fatty acid modification on the flavor and oxidative stability of fresh pork needs to be evaluated.

The use of hexanal content as an indication of the extent of lipid oxidation is not limited to meat and meat products. Measurement of hexanal content has been used to monitor the development of lipid oxidation during storage or processing in a range of foods (Table 2.2). In some cases, hexanal was used in combination with a second volatile compound to monitor lipid oxidation and resultant flavor quality. Pentanal and hexanal were used to assess flavor quality of vegetable oils (Warner et al., 1978) and potato chips (Jeon and Bassette, 1984). Both studies concluded that hexanal alone was as useful an indicator as the pair. Octanal and hexanal were used to assess oxidation of hazelnuts (Kinderlerer and Johnson, 1992). Octanal was selected as an indicator due to the high amount of oleic acid in hazelnut oil. While the content of both compounds increased during storage, hexanal content increased more rapidly. Bengtsson et al. (1967) used measurement of ethanol and hexanal as well as sensory evaluation to assess oxidation in peas subjected to different harvesting conditions, then blanched and frozen. Ethanol was selected as a indicator compound because its presence indicates enzyme activity while

Table 2.2 Studies using hexanal measurement to assess lipid oxidation in foods during processing or storage

Food	Stage Monitored	Reference
Cookies	Storage	Lingnert (1980)
Brown rice	Storage	Shin et al. (1986)
Hazelnuts	Storage	Kinderlerer and Johnson (1992)
Milk base, spray-dried	Storage	Roozen and Linssen (1992)
Milk powder, whole	Storage	Hall and Andersson (1985)
Oat breakfast cereal	Storage	Fritsch and Gale (1977)
Oat products	Processing	Ekstrand et al. (1993)
Oatmeal	Storage	Guth and Grosch (1994)
Peanut paste	Processing	Muego-Gnanasekharan and Resurreccion (1993)
Peanuts	Storage	Bett and Boylston (1992)
Peas	Processing	Bengtsson et al. (1967)
Pecans	Storage	Erickson (1993)
Pinto bean, dehydrated	Storage	Hartman et al. (1994)
Potato chip	Storage	Jeon and Bassette (1984)
Potato flakes	Storage	Sapers et al. (1972)
Potato granules	Processing	Hallberg and Lingnert (1991)
Potato granules	Storage	Boggs et al. (1963)
Potatoes, puffed	Storage	Konstance et al. (1978)
Soybeans	Processing	Moreira et al. (1993)
Vegetable oil	Storage	Warner et al. (1978)

hexanal is formed either enzymatically or via autoxidation (Bengtsson et al., 1967). Both ethanol and hexanal content corresponded with sensory results during post-harvest storage at 20°C. However, only hexanal content reflected sensory scores during frozen storage since hexanal continued to form while ethanol did not.

The ultimate criterion of suitability of a compound as an index of lipid oxidation for foods is adequate correlation with sensory data (Shahidi, 1994). Hexanal has been shown to relate well to off-flavor development in various foods during storage. Correlation coefficients have been reported between sensory scores and hexanal content for cooked and stored broiler breast and thigh (Ang and Lyon, 1990), ground beef (St. Angelo et al., 1987), beef slices (Hwang et al., 1990), ground pork (Shahidi et al., 1987), as well as oat breakfast cereal (Fritsch and Gale, 1977) with values of 0.92 and 0.84, 0.80, 0.89, 0.98, and 0.99, respectively.

2.5 Measurement of Lipid Oxidation

A uniform and satisfactory method to evaluate flavor quality and stability of lipid foods needs to be developed (Min, 1981). When evaluating the usefulness of an analytical procedure, three criteria should be considered: 1) the representativeness of the parameter to be measured--does it consistently occur in oxidizing systems and does the degree to which it occurs correspond to the extent of oxidation; 2) the specificity of the parameter for lipid oxidation; and, 3) the specificity of the method for that parameter (Gray, 1978).

The methods used to measure the degree of lipid oxidation in foods can be grouped into two categories based on the changes occurring in the oxidizing system. Changes due to the primary reaction of lipid oxidation include hydroperoxide formation,

measured and expressed as the peroxide value (PV) and oxygen absorption. Changes due to the secondary reactions of lipid oxidation include malonaldehyde formation, traditionally measured by the thiobarituric acid (TBA) test, and volatile compound formation, measured by gas chromatography (GC). These methods can be ranked by their respective predictive values for stability, shelf-life, and consumer acceptability of the product, in decreasing order, as sensory evaluation, headspace volatiles, oxygen absorption, PV, and TBA-reactive substances (RS) (Frankel, 1993).

In general, measurement of the primary changes is useful only in the initial stages of lipid oxidation (Pearson et al., 1977; Melton, 1983; Ajuyah et al., 1993; Rossell, 1994). Hydroperoxides are tasteless and odorless and, therefore, do not contribute to rancid flavor (Prior and Loliger, 1994). As intermediates, the peroxide concentration will decrease as oxidative deterioration worsens. This may result in an underestimation of the degree of oxidation (Gray and Monahan, 1992), particularly during storage (Shahidi, 1994). Another drawback is that the fat extraction step, necessary in the analysis of foods, may cause the formation of additional peroxides or the decomposition of the existing peroxides prior to PV determination (Rossell, 1994). Oxygen absorption is measured indirectly as the loss of oxygen in the headspace above a sample in a sealed container. Oxygen absorption methods have limited sensitivity and therefore require a high degree of oxidation to detect a change (Frankel, 1993; Prior and Loliger, 1994). In addition, oxygen absorption may occur during protein oxidation, as well as during lipid oxidation, and is therefore not specific (Melton, 1983).

The secondary reactions result in formation of compounds which will affect the flavor profile of the oxidizing food system. Sensory evaluation is the most important and

common way to determine flavor quality (Min, 1981) since odor and flavor evaluations relate directly to consumer acceptance (Frankel, 1993). However, sensory evaluation is time-consuming, expensive (Min, 1981) and the quality of the data is highly dependent on the quality of training received by the panelists (Frankel, 1993) since recognizing and quantifying lipid oxidation off-flavors requires both good taste acuity and considerable experience (Fritsch, 1994).

The TBA test is the oldest and most frequently used test for assessing lipid oxidation in muscle foods (Melton, 1983; Shahidi, 1994; Prior and Loliger, 1994) and other biological systems (Gray and Monahan, 1992). The test is simple and sensitive, resulting in the formation of a red chromophore measurable spectrophotometrically at 532-535 nm or by high-pressure liquid chromatography (HPLC) or GC. It has several limitations. First, malonaldehyde is a relatively minor lipid oxidation product arising only from fatty acids with three or more double bonds (Gray and Monahan, 1992; Prior and Loliger, 1994). Therefore, it is not suitable for the measurement of oxidation products of foods containing mainly oleic acid with one double bond and linoleic acid with two double bonds (Frankel, 1993). Second, the formation of malonaldehyde does not always result in increased TBA values in stored muscle foods since the compound itself may react further (Igene et al., 1985; St. Angelo, 1996). Third, it is not specific for lipid oxidation since other food components also react with TBA (Prior and Loliger, 1994) and may result in an overestimation of the extent of oxidation (Frankel, 1993). This limitation is reflected in the common use of the term "thiobarbituric acid-reactive substances" suggesting that other materials are measured in addition to malonaldehyde. Fourth, the amount of chromophore formed and detected varies depending on the protocol used (St. Angelo,

1996). Therefore, the TBA test should be used in combination with a complementary procedure, such as hexanal measurement, and/or frequently compared to trained sensory panel results (Igene et al., 1985; Gray and Monahan, 1992; Prior and Loliger, 1994).

Analysis of volatile compounds by GC is closely related to flavor evaluation and therefore is the most suitable method for comparison with the results of sensory panel tests (Frankel, 1993). In fact, GC is the most commonly used technique for flavor analysis (Reineccius, 1996). Due to its high resolution, GC is the most powerful tool available for determining volatile oxidation products (Prior and Loliger, 1994). However, sample preparation procedures used to obtain sufficient amounts of the compounds to be instrumentally detectable usually cause qualitative and quantitative compositional changes in the sample (Jennings and Filsoof, 1977). Isolation of the volatile compounds prior to GC analysis is typically achieved by one of three techniques, or variation of these: simultaneous distillation-extraction (SDE), static headspace, and dynamic headspace (also called purge-and-trap). The SDE technique involves distillation of an aqueous solution of the food and simultaneous extraction of the distillate with solvent. It is used extensively for flavor extraction. It is used less widely for oxidation determination of foods because of possible destruction of volatiles during distillation, loss of volatiles during concentration, and coelution of some compounds with the solvent (Prior and Loliger, 1994). Static headspace sampling involves direct injection of the gases above a food sample sealed in an airtight container onto a GC for analysis and requires virtually no sample preparation. This technique is relatively simple, reproducible, and rapid (Reineccius, 1996) and most accurately represents the flavor/odor above a food; however, sensitivity is a problem (Prior and Loliger, 1994) since insufficient quantities of

compounds may not permit accurate and precise quantitation (Reineccius, 1996). Dynamic headspace involves purging the volatiles from a sample by a stream of inert gas and trapping them with an adsorbent material. Because volatiles from large amounts of sample can be concentrated, the sensitivity is better than that of the static headspace technique. However, several parameters will affect the performance of a purge-and-trap system: sample temperature; length of time of volatile collection; nitrogen flow rate; sample vessel geometry; use of vacuum; adsorbent material type; amount of adsorbent; and sample sparging versus headspace purging (Vercellotti et al., 1992). Differences in the relative affinities for an adsorbent and the vapor pressures of volatile compounds can result in either breakthrough losses on trapping or poor recoveries (Vercellotti et al., 1992). Recovery of volatile compounds from the adsorbent can be accomplished by either thermal desorption or solvent elution. While thermal desorption is faster than solvent elution, the heat may alter or destroy some volatile compounds (Prior and Loliger, 1994). Thermal desorption and associated equipment, in general, is complex and costly compared to materials needed for solvent elution (Olafsdottir et al., 1985). However, depending on the chromatograph conditions and the solvent used, early-eluting compounds may be lost in the solvent peak (Jennings and Filsoof, 1977). While clearly no single sample preparation technique is uniformly satisfactory, a given procedure may be better for a particular compound in a particular sample (Jennings and Filsoof, 1977).

Another drawback of GC headspace analysis is its restriction to measurement of volatile compounds ignoring the contributions of non-volatile compounds to flavor (Jennings, 1977). A survey of the literature indicates that hexanal binding has been observed in numerous protein systems including: soy protein (Arai et al., 1970; Franzen

and Kinsella, 1974), soy protein isolate (Franzen and Kinsella, 1974; Gremli, 1974), soy glycinin and beta-conglycinin (O'Keefe et al., 1991a, b), lysozyme (Funes et al., 1980; Tashiro et al., 1985; Okitani et al., 1986), whey-based and egg-based fat replacers (Schirle-Keller et al., 1992; Schirle-Keller et al., 1994), alpha-lactalbumin, bovine serum albumin (BSA), leaf protein concentrate, single-cell protein, textured vegetable protein (Franzen and Kinsella, 1974), myosin and actin (Gutheil and Bailey, 1992). Gremli (1974) developed an analytical method using a high vacuum-shell freezing system to determine the amount of a flavor compound present in and "retained" by a protein solution preventing volatilization. By comparing measurements taken using this method to those obtained using an ambient headspace procedure and using a solution devoid of protein as the control, the percentages of flavor compounds "reversibly bound" and "irreversibly bound" by the protein were calculated. For a solution containing 5% soy protein, the amounts of hexanal reversibly and irreversibly retained were 37-44% and <5%, respectively. To measure the concentration of protein-bound carbonyl compounds present in a solution, Franzen and Kinsella (1974) used a variety of proteins to survey the extent of nonvolatilization of various carbonyl compounds. The concentration of volatile carbonyl compound over a carbonyl-protein solution was measured by headspace GC and compared to control solutions devoid of protein. The concentration ratios of hexanal in various protein solutions suggested that 10-21% of the hexanal added to the solution was retained by the protein. Protein-bound hexanal has been measured directly by enzyme assay (Chiba et al., 1979). Aldehyde dehydrogenase purified from bovine liver mitochondria was found to convert soy protein-bound aldehyde and free aldehyde to alcohol irrespective of carbon chain length and at comparable rates. Using a second

enzyme, alcohol dehydrogenase, which does not act on protein-bound aldehyde, total and free aldehyde content can be measured and bound aldehyde content determined by difference.

While it is extremely doubtful that any instrumental method will replace human sensory evaluation of foods in the foreseeable future, various techniques can be used as screening procedures and to supplement sensory analysis (Reineccius, 1996). Despite the limitations, the purge-and-trap technique has been used for many quality control methods (Vercellotti et al., 1992) and solvent extraction from a Tenax adsorbent has become popular in research studies (Reineccius, 1996). However, further use of GC outside the research laboratory is hindered by time limitations and method complexities (Reineccius, 1996).

2.6 Immunoassays

The basis of all immunoassays is the highly specific immunological interaction between an antibody and a corresponding antigen (Hefle, 1995). Currently, the most common type of immunoassay is the enzyme-linked immunosorbent assay (ELISA) (Fukal, 1991). Amplification is achieved by using a detector system consisting of an enzyme label plus substrate. Detection is indicated by color development and quantitation correlates to color intensity. Use of a competitive format allows quantitation of a particular analyte in an unknown concentration in a sample (Figure 2.3).

Immunoassays are a rapid, cost-effective, and easy-to-use alternative to conventional analytical techniques achieving sensitivity and specificity without requiring highly trained analysts or sophisticated equipment (Samarajeewa et al., 1991). In addition,

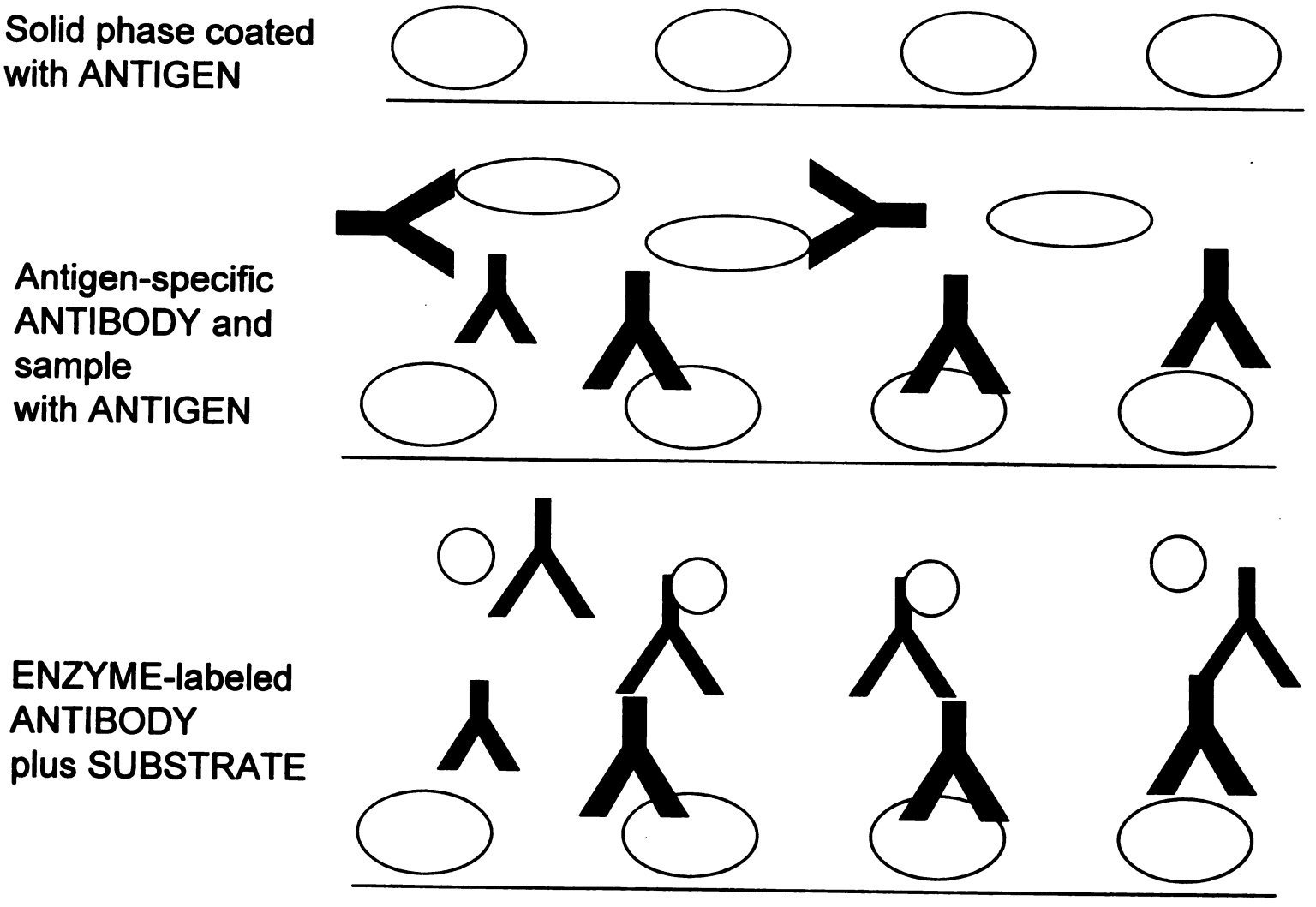


Figure 2.3 Competitive indirect enzyme-linked immunosorbent assay

immunoassays permit simultaneous testing of many samples. Commercially available immunoassay kits are portable and can be used for routine surveillance in a processing facility or retail outlet. As an analytical tool, immunoassays have already proven to be very useful for the assessment of a variety of substances in meat products, including detection of pesticides, drugs, microorganisms, and microbial toxins (Fukal, 1991). To improve the quality and safety of meat and poultry products, ELISAs have been developed to monitor thermal processing, to detect species adulteration, non-meat protein addition, various contaminants and residues, and to diagnose animal diseases (Fukal, 1991; Smith, 1995). Immunoassays developed to monitor endpoint cooking temperature replace assays based on protein solubility, enzyme activity, color, and electrophoretic patterns which are often imprecise and difficult to interpret (Smith, 1995). ELISA kits using antibodies to species-specific serum proteins have been commercialized for detection of species adulteration of as many as 11 species by one assay (Smith, 1995). Meat sample preparation may consist of as little as a saline extraction and dilution of the extract (Fukal, 1991).

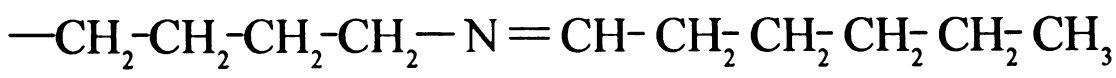
Antibody production requires an immunogenic molecule to elicit an immune response in the animal system. Haptens, substances possessing a molecular weight of less than 1,000 daltons, are not immunogenic and must be chemically bound to a carrier protein to function as immunogens (Hefle, 1995). Protein modifications as simple as ethylation and methylation have resulted in production of specific antibodies against the modified protein and not to epitopes of the native protein (Steinbrecher et al., 1984). Anti-aldehyde antibodies have already been developed to formaldehyde (Steinbrecher et al., 1984), acetaldehyde (Steinbrecher et al., 1984; Israel et al., 1986; Klassen et al., 1990;

Niemela et al., 1991; Perata et al, 1992; Worrall et al., 1991; Israel et al., 1992; Klassen et al., 1994; Thiele et al., 1994), malonaldehyde (Haberland et al., 1988; Palinski et al., 1989; Palinski et al., 1995; Preobrazhensky et al., 1995), and 4-hydroxynonenal (Palinski et al., 1989; Petit et al., 1995, Uchida et al., 1995). Petit et al. (1995) proposed the use of an immunoassay incorporating anti-malonaldehyde (MA) antibodies for measuring MA-induced cell membrane alterations resulting from lipid oxidation. Niemela (1993) developed an immunoassay incorporating anti-acetaldehyde (AA) antibodies for detection of AA-adducts and proposed its use for diagnosis of excessive alcohol consumption prior to the appearance of clinical signs of chronic alcoholism.

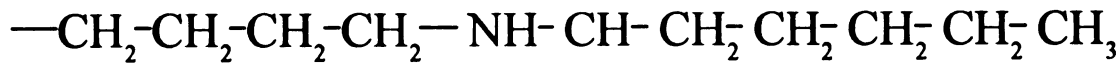
2.7 Epitope Structure

Numerous studies have demonstrated covalent binding between aldehydes and proteins occurs in vivo and that the adducts can be measured by immunological methods (Curtiss and Witztum, 1983; Israel et al., 1986; Niemela et al., 1991; Worrall et al., 1991; Palinski et al., 1995; Preobrazhensky et al., 1995; Yoritaka et al., 1996). Modifications altering protein structure as little as methylation can render endogenous proteins immunogenic (Steinbrecher et al., 1984).

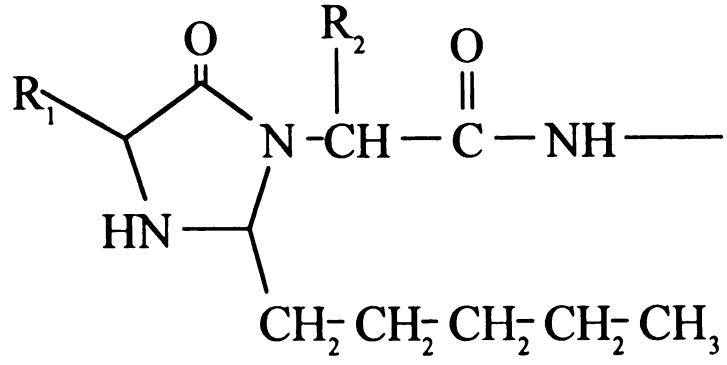
The products of aldehyde-protein reactions differ in mechanism of formation and structure depending on the reaction conditions and can be grouped into three categories: unstable, stable-reduced, and stable-nonreduced (Figure 2.4). The "unstable" adducts are Schiff bases, resulting from reaction between carbonyl carbons and free amino groups. These products form relatively quickly (San George and Hoberman, 1986) and readily dissociate when exposed to dialysis, gel filtration, or treatment with weak acid or base



UNSTABLE ADDUCT (SCHIFF BASE)



STABLE REDUCED ADDUCT



STABLE NONREDUCED ADDUCT (2-methyl imidazolidin-4-one)

Figure 2.4 Structures of three types of products resulting from hexanal-protein reactions: Unstable (Schiff base), stable-reduced, and stable-nonreduced

(Donohue et al., 1983; Tuma et al., 1984). These Schiff bases can be stabilized by reduction to secondary amines using a reducing agent. The resultant "stable-reduced" products are relatively irreversible and generally withstand exhaustive dialysis and gel filtration (Klassen et al., 1994). Because the reduced products are also stable to denaturation and enzymatic and acid hydrolysis, the binding sites can be identified by amino acid analysis (San George and Hoberman, 1986). "Stable-nonreduced" adducts are also stable to prolonged dialysis but are distinctly different from those produced under reducing conditions (Klassen et al., 1994). Binding occurs initially between an aldehydic carbonyl and an amino group of a protein forming a Schiff base as before. According to San George and Hoberman (1986), the amino group in the protein terminus and the Schiff base intermediate is stabilized by binding of the carbonyl carbon with the nitrogen of the peptide bond between the N-terminal and adjoining amino acid residue (San George and Hoberman, 1986). The resultant structure is a stable 5-membered ring, or imidazolidinone derivative.

The structural differences of protein-aldehyde adducts formed under differing reaction conditions is supported by several immunological studies. Antibodies, based on epitope specificities, are able to distinguish aldehyde-modified proteins prepared under reducing condition from those prepared under non-reducing conditions (Klassen et al., 1990; Klassen et al., 1994; Thiele et al., 1994). The effect of reaction conditions on the type and amounts of adducts formed was clearly demonstrated in a study of nonenzymatic glucosylation. Curtiss and Witztum (1983) developed an immunoassay to measure glucitollysine, the reduced hexose alcohol form of glucose conjugated to the ε-amino group of lysine. Glucose and low-density lipoprotein (LDL) were reacted under differing

conditions and the amounts of glucitollysine formed were compared. In the absence of a reducing agent, glucose formed a labile intermediate Schiff base with the ε-amino group of lysine which underwent an Amadori rearrangement to form a relatively stable ketoamine which equilibrated with its hemiketal or ring form. When glucosylation is carried out in the presence of sodium cyanoborohydride ($NaCNBH_3$), the labile Schiff base is immediately and quantitatively reduced to glucitollysine. When sodium borohydride ($NaBH_4$) was used as the reducing agent, Schiff base, ketoamine, and hemiketal products are all reduced, resulting in the formation of glucositollysine and mannositollysine, its epimer. The application of these findings was as follows. Glucosylated proteins and lipoproteins have been implicated in diabetes-related atherosclerosis. The concentrations of glucitollysine residues were found to be threefold higher in diabetic total plasma proteins and isolated lipoproteins compared to nondiabetic proteins. Since the adducts formed in vivo were mostly Amadori forms and, therefore, not recognized by the antibody, pretreatment of the plasma with each reducing agent was required and the difference in glucitollysine contents taken. An immunoassay for the quantitative assessment of glycitollysine content should prove useful.

The type of the reducing agent also influences the adduct structure. Tuma et al. (1987) attempted to characterize, by high-performance liquid chromatographic analysis, the stable adducts formed through reactions of BSA and radiolabelled acetaldehyde using three different reducing agents: $NaBH_4$, $NaCNBH_3$, and ascorbate. Each reaction generated a different elution pattern. When $NaBH_4$ was used as the reducing agent, four different types of adducts were found, including ethyllysine. When $NaCNBH_3$ was used instead, the ethyllysine structure predominated with only minor amounts of other adducts.

These findings can be explained by the fact that $NaCNBH_3$ readily and selectively reduces Schiff bases giving a higher yield of modified lysines than $NaBH_4$ which, in addition to reducing Schiff bases, also reduces aldehydes and ketones to alcohols (Jentoft and Dearborn, 1979). Ascorbate, a physiological reducing agent, was found to stabilize acetaldehyde-BSA adducts at concentrations comparable to those found in the liver (Tuma et al., 1984). However, none of the four different adduct structures formed in the presence of ascorbate were ethyllysine (Tuma et al., 1987). Tuma et al. (1987) also studied non-reducing conditions and the adducts formed using polylysine instead of BSA under each of the scenarios discussed. Overall, of the six different adduct structures detected, only one could not positively be identified as a lysine derivative.

Other studies have also indicated that lysine is the major amino acid participating in the binding of aldehyde to protein (Table 2.3). Jentoft and Dearborn (1979) reported that arginine, histidine, methionine, and tryptophan do not form stable derivatives with aldehyde under conditions of reductive alkylation or do so very slowly.

Table 2.3. Studies characterizing the dominant epitope of antibodies against immunogens formed by reaction of aldehyde and protein using sodium cyanoborohydride as reducing agent.

Reaction	Reactant[a]	Epitope[b]	Reference
Methylation	Formaldehyde	Dimethyllysine	Steinbrecher et al. (1984)
Ethylation	Acetaldehyde	Methyllysine	Steinbrecher et al. (1984)
Ethylation	Acetaldehyde	Ethyllysine	Israel et al. (1986), Tuma et al. (1987), Thiele et al. (1994)
Alkylation	Malonaldehyde	Malonaldehyde-lysine	Palinski et al. (1989)
Alkylation	4-Hydroxynonenal	4-Hydroxynonenal-lysine	Palinski et al. (1989)
Glucosylation	Glucose	Glucitollysine	Curtiss and Witztum (1983)
Alkylation	Glyoxylic acid	Carboxymethyllysine	Ikeda et al. (1996)

[a] Used to modify the protein
[b] Based on cross-reactivity experiments

CHAPTER 3

PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES TO HEXANAL-LYSINE ADDUCTS

3.1 ABSTRACT

Hexanal content is a popular index of lipid oxidation in foods, including muscle tissue. Methods currently available to monitor oxidation of unsaturated fatty acids, including the thiobarbituric acid-reactive substances (TBA-RS) assay, are less than optimal, particularly in a complex matrix such as meat. The purpose of this study was to develop and characterize hexanal-specific polyclonal antibodies and incorporate these into an enzyme-linked immunosorbent assay (ELISA). Because free hexanal is nonimmunogenic, it was covalently attached to a carrier-protein prior to immunization. Using sodium cyanoborohydride to form stable conjugates, hexanal was reacted with bovine serum albumin (BSA), for use as the immunogen, and to chicken serum albumin (CSA) for use as ELISA solid phase. The degree of modification of BSA with hexanal was determined by measuring free amino groups by trinitrobenzene sulfonic acid (TNBS) assay and complete loss of lysine residues was observed following reductive alkylation. Polyclonal antibodies produced in rabbits immunized with hexanal-modified BSA had antiserum titers as high as 6.15×10^{-5}. A competitive indirect ELISA was developed to evaluate antisera reactivity

by CI-ELISA. The immunoassay was sensitive with a detection limit of 1.5 ng/mL hexanal and precise with a mean coefficient of variation of 9.3% (n = 281). The antibodies were found to be carrier-independent, reacting with hexanal-BSA, hexanal-CSA and hexanal-KLH conjugates but not the corresponding unmodified proteins. Aldehyde specificity was evaluated using CSA-conjugated alkanals of 3-9 carbons. Cross-reactivity with n-heptanal, n-pentanal, n-octanal and 2-methylbutanal was determined to be 86.3%, 11.8%, 2.2%, and 1.1%, respectively. The antibodies reacted strongly with hexanal-modified lysine (α-, ε-diaminocaproic acid) and very strongly with hexanal-modified ε-aminocaproic acid. The antibodies did not recognize free amino acids or free hexanal. The immunodominant epitope is proposed to be hexanal-modified lysine.

3.2 INTRODUCTION

Lipid oxidation leads to the destruction of lipids in biological and food systems (Kappus, 1991). A number of diseases have been related to the induction of lipid peroxidation, including cancer (Kappus, 1991), rheumatoid arthritis (Lopez-Bote et al., 1993) and Parkinson's disease (Yoritaka et al., 1996). Evidence indicating that oxidized low density lipoprotein (LDL) contributes to atherogenesis is substantial (Palinski et al., 1995). Rancidity arising from lipid oxidation is often the decisive factor in determining the useful storage life of food products (Frankel, 1993). Unfortunately, methodological problems have severely limited efforts to achieve a clearer understanding of lipid oxidation and its effects (Gutteridge and Halliwell, 1990); however, development of improved analytical procedures will lead to great advancement in the knowledge of lipid oxidation (Kappus, 1991).

The most widely used assay for measuring lipid peroxidation is the 2-thiobarbituric acid (TBA) test (Gutteridge and Halliwell, 1990; Gray and Monahan, 1992). While this test is sensitive for oxidation products of fatty acids containing three or more double bonds, it is not suitable for oxidation products of oleic and linoleic acids (Frankel, 1993). Another limitation of the assay is its lack of specificity since many other compounds are also TBA-reactive, including amino acids and carbohydrates (Gutteridge and Halliwell, 1990). Many techniques have been used to measure lipid oxidation and evaluate oxidative stability, each with limitations which have been previously reviewed (Gray, 1978; Melton, 1983; Prior and Loliger, 1994; Rossell, 1994; Shahidi, 1994). One approach that has been successfully used to follow lipid oxidation in both biological specimens and food systems is hexanal measurement by headspace gas chromatography (Shahidi, 1994). Hexanal is an important oxidative decomposition product of omega-6 polyunsaturated fatty acids (Frankel and Tappel, 1991) and is one of the compounds contributing to the undesirable odor and flavor associated with rancidity in food products (Pearson etal., 1977). Static headspace sampling involves direct injection of the gases above a sealed sample onto a gas chromatograph (GC) for analysis and requires virtually no sample preparation. This technique has been used successfully in oxidative susceptibility studies to measure hexanal in the headspace of red blood cell membranes (Frankel and Tappel, 1991), of liver homogenate (Frankel et al., 1989) and of plasma lipoproteins (Walzem et al., 1995). While this technique is relatively simple, rapid, and reproducible, it lacks the sensitivity necessary to detect compounds present in small quantitites (Reineccius, 1996). Dynamic headspace methods, which involve purging volatile compounds from a sample by a stream of inert gas and trapping them with an adsorbent material, have been developed to

improve sensitivity. However, several parameters will affect the performance of a dynamic headspace or purge-and trap system, including sample temperature, length of time of volatile collection, carrier gas flow rate, sample vessel geometry, and type and amount of adsorbent (Vercellotti et al., 1992). Currently, neither a standard method for the quantitation of hexanal nor a convention for the reporting of hexanal data exist, making comparison of results difficult. Hexanal-protein binding (Franzen and Kinsella, 1974; Gremli, 1974; Esterbauer et al., 1987; O'Keefe, 1991a,b; Gutheil and Bailey, 1992; Schirle-Keller et al., 1992; Holley et al., 1993; Schirle-Keller et al., 1994) further complicates accurate quantitation by headspace gas chromatographic methods.

Immunoassays are increasing in popularity because they offer both analytical and cost-savings benefits over conventional techniques, including sensitivity, specificity, speed, and simultaneous testing of many samples (Fukal, 1991; Samarajeewa et al., 1991; Deshpande, 1994; Hefle, 1995). Protein modifications as simple as ethylation and methylation have resulted in production of specific antibodies against the modified protein and not to epitopes of the native protein (Steinbrecher et al., 1984). Anti-aldehyde antibodies have already been developed to formaldehyde (Steinbrecher et al., 1984), acetaldehyde (AA) (Steinbrecher et al., 1984; Israel et al., 1986; Klassen et al., 1990; Niemela et al., 1991; Perata et al., 1991; Worrall et al., 1991; Israel et al., 1992; Klassen et al., 1994; Thiele et al., 1994), malonaldehyde (MA) (Haberland et al., 1988; Palinski et al., 1989; Chancerelle et al., 1991; Palinski et al., 1995; Preobrazhensky et al., 1995), and 4-hydroxynonenal (Palinski et al., 1989; Petit et al., 1995; Yoritaka et al., 1996). Petit et al. (1995) proposed the use of an immunoassay incorporating anti-MA antibodies for measuring MA-induced cell membrane alterations resulting from lipid oxidation. Niemela

(1993) developed an immunoassay incorporating anti-AA antibodies for detection of AA-adducts and proposed its use for diagnosis of excessive alcohol consumption prior to the appearance of clinical signs of chronic alcoholism. In food, Jourdan et al. (1984) developed an ELISA to measure limonin, a bitter triterpenelactone, in citrus juice.

The primary goal of this study was to produce and characterize sensitive polyclonal antibodies specific to hexanal to be incorporated into an enzyme immunoassay. Our hypothesis was that antibodies to hexanal could be produced and would recognize a derivatized lysine epitope. The specific objectives were three-fold. First, because hexanal is a hapten and non-immunogenic itself, it had to be covalently bound to a carrier protein. It was critical to attach as many hexanal molecules as possible to the carrier protein, presumably via the ε-amino group of the lysine side chains, to ensure all protein fragments appearing on the surface of antigen-presenting cells contained hexanal. Second, following immunization of rabbits with hexanal-modified BSA, it was necessary to develop an ELISA to determine sensitivity and specificity of the resultant polyclonal antibodies. Third, it was important to characterize the antibodies for protein, aldehyde and amino acid specificities by assessing antibody cross-reactivity with compounds of related and/or relevant structure to that of the antigen.

3.3 MATERIALS & METHODS

3.3.1 Protein-Hapten Conjugation by Reductive Alkylation. Alkylation of proteins and amino acids was performed by the method of Steinbrecher et al. (1984) with these modifications: 1) aldehyde was added in excess to obtain maximum modification, 2) aldehyde was added immediately prior to reducing agent, and 3) reaction was run at

ambient temperature and a basic pH to accelerate the reaction (Jentoft and Dearborn, 1979). Specifically, an aliquot of 246 μL of hexanal (H-9008, Sigma Chemical Co., St. Louis, MO) was added to 9 mL of 16.7 mg/mL bovine serum albumin (BSA; A-7511, Sigma) diluted in phosphate-buffered saline (PBS; 0.1M sodium chloride, 0.01M sodium phosphate, pH 7.4) and the solution vortexed. Reducing conditions at pH 8.5-9.0 were established by adding 1 mL of 800 mM sodium cyanoborohydride ($NaCNBH_3$; S-8628, Sigma) dissolved in 0.1 N NaOH to the aldehyde-protein mixture. The resultant reaction concentrations were: 200 mM aldehyde, 15 mg/mL protein, and 80 mM cyanoborohydride. The solution was held at ambient temperature for 12 hr before dialyzing overnight against two changes of PBS. The same protocol was used to modify keyhole limpet hemocyanin (KLH; H-7017, Sigma), chicken serum albumin (CSA; A-3014, Sigma), and a high molecular weight standard protein mixture (SDS-6H, Sigma) with hexanal. The standard contained 6 proteins with molecular weights ranging from 29-205 kD. The molecular weight standard was reconstituted by adding 1.8 mL PBS (pH 7.4), 100 μL of 10% sodium dodecyl sulfate (SDS; 28364, Pierce, Rockford, IL) and 100 μL of 0.1 N HCl and standing at ambient temperature overnight.

CSA was also modified with other aldehydes, ketones and alcohols: propanal (propionaldehyde; P-6889, Sigma), butanal (butyraldehyde; B10,328-4, Aldrich Chemical Co., Milwaukee, WI), pentanal (valeraldehyde; V-2378, Sigma), heptanal (heptaldehyde; H212-0, Aldrich), octanal (O-7128, Sigma), nonanal (nonyl aldehyde; N3,080-3, Aldrich), trans-2-hexenal (H-7383, Sigma), 2-methylpentanal (25,856-3, Aldrich), 2-methylbutanal (M3,347-6, Aldrich), 3-methylbutanal (isovaleraldehyde; 14,645-5, Aldrich), and 2-heptanone (12,336-6, Aldrich). CSA was carried through the reductive alkylation

procedure with the alcohols, 2-hexanol (12,857-0, Aldrich) and n-hexanol (hexyl alcohol; H1,330-3, Aldrich) substituted for an aldehyde, as described above.

Hexanal-modified amino acids were prepared as described above, except that the reaction concentrations were 200 mM hexanal, 20 mM sodium cyanoborohydride and 200 mM amino acid, and dialysis was not performed due to the small size of the molecules. The amino acids were L-lysine monohydrochloride (L-5626, Sigma), D-lysine monohydrochloride (L-5876), L-arginine (A-5006, Sigma), glycine (15527-013, Life Technologies, Inc., Gaithersburg, MD), and ε-amino-n-caproic acid (A-7824, Sigma). Octanal was substituted for hexanal for reductive alkylation with γ-amino-n-butanoic acid (GABA; A-2129, Sigma).

The degree of modification achieved in each reaction was determined by measuring the loss of reactive amino groups by the trinitrobenzenesulfonic (TNBS) acid assay of Habeeb (1966). Briefly, 1 mL of protein solution, containing approximately 1 mg/mL protein, was added to 1 mL of 4% sodium bicarbonate ($NaHCO_3$; 7412, Mallinckrodt, Paris, KY) buffer, pH 8.5, and 1 mL of 0.1% TNBS (picrylsulfonic acid; P-2297, Sigma). The solution was vortexed and incubated for 2 hr in a 40°C water bath. Following incubation, 1 mL of 10% SDS was added and the solution vortexed to solubilize the protein. Finally, 0.5 mL of 1 N HCl was added and the solution vortexed. The absorbance was read at 335 nm. The results were determined from a standard curve of leucine solutions ranging in concentration from 10-300 μM and expressed as moles reactive amino groups per mole protein (Adler-Nissen, 1979).

Amino acid analysis was performed by the method of Heinrikson and Meredith (1984) using high performance liquid chromatography following sample derivatization of

native and hexanal-modified CSA with phenylisothiocyanate. The results were expressed as a molar percentage for each amino acid.

3.3.2 Protein Labeling by Reductive Alkylation. The number of adducts formed between hexanal and CSA was determined by measuring the incorporation of tritium from radiolabeled sodium cyanoborohydride ([^{3}H]NaCNBH$_3$; EC5911, Amersham Corp., Arlington Heights, IL) (Klassen et al., 1990). CSA (1.5 mg) and hexanal (2.5 μL) were combined with 87.5 μL of PBS. The protein solution and 10 μL of 0.1 N NaOH were added directly to the vial containing the isotope to prevent unnecessary handling and loss. The reactant concentrations were 15 mg/mL CSA, 200 mM hexanal, and 4 mM [^{3}H]NaCNBH$_3$. The reaction was allowed to continue for 12 hr at ambient temperature. The solution was then dialyzed using a Slide-A-Lyzer cassette (66415, Pierce Chemical Co., Rockford, IL) at ambient temperature against 3 changes of 150 mL of PBS over 24 hr. The solution was diluted 1:100 in PBS and a 10 μL aliquot was combined with 4 mL of scintillation fluid (Safety-Solve, Research Products International Corp., Mt. Prospect, IL). Radioactivity was measured using a scintillation counter (Packard 4430, United Technologies, Downers Grove, IL). The number of adducts formed was expressed as moles tritium incorporated per mole protein.

3.3.3 Polyclonal Antibody Production. Three 3-month old, female White New Zealand rabbits (Hazelton Laboratories, Kalamazoo, MI), identified as #45903, 45904, 45911, were housed by the Michigan State University Laboratory Animal Resources and handled according to the Rabbit Antibody Production Service (RAPS) program. Each rabbit was immunized subcutaneously with a total of 1 mL of antigen-adjuvant emulsion administered in 8-10 sites on its back. The initial injection consisted of 500 μg of hexanal-modified

BSA in 0.5 mL of 0.85% sterile saline emulsified with 0.5 mL of Freund's Complete adjuvant (F-5881, Sigma) per rabbit. Emulsification was carried out according to the procedure of Desrocher (1994). At total volume of 4 mL of antigen solution was prepared to allow for losses during emulsification and injection. Booster injections were administered at 4, 8, and 18 weeks and consisted of 500 μg of hexanal-modified BSA emulsified with Freund's Incomplete adjuvant (F-5506, Sigma). Each rabbit was bled from the marginal ear vein prior to the initial injection to obtain control (preimmune) serum and 10 days following each injection to check serum titers.

The sera were fractionated using saturated ammonium sulfate (SAS; pH 7.6) according to a modification of the method of Harlow and Lane (1988). Briefly, the blood was allowed to coagulate at ambient temperature for 2 hr and the clot to contract at 4°C overnight. Next, the blood was decanted into a centrifuge tube and spun at 10,000 x g for 20 min at 4°C. Saturated ammonium sulfate (SAS; pH 7.6) was added dropwise to the supernatant to achieve 30% saturation (Hebert et al., 1973). The solution was centrifuged at 10,000 x g for 20 min at 4°C, the pellet dissolved to the original volume in PBS, diluted 2:1 with SAS, and the solution stirred at room temperature for 30 min; this was performed twice. The solution was centrifuged a final time and diluted in PBS to half the original volume before dialyzing at 4°C against 4 L PBS for 3 days, changing the buffer every 24 hr. The serum was diluted to the original serum volume and frozen for storage.

3.3.4 Indirect ELISA for serum titer determination. Serum titers for each bleeding were determined by indirect ELISA. The solid phase support was a 96-well microtiter plate consisting of disposable Immunlon 2 Removawell strips (011-010-6302, Dynatech Laboratories, Inc., Chantilly, VA) fitted into a Removawell strip holder (011-010-6604,

Dynatech). Each plate was coated with 100 μL per well of 2 μg/mL hexanal-modified CSA in 0.1M carbonate buffer (pH 9.6) and held at 4°C for 16 hr to ensure binding, then washed four times with PBS-Tween (0.1 M sodium chloride, 0.015 M sodium phosphate, 0.05 % polyoxyethylenesorbitan monolaurate, Tween 20; P-1379, Sigma) using an automatic plate washer (Model ELP-40, Bio-Tek Instruments, Inc., Winooski, VT). Each plate was pretreated with 300 μL per well of PBS-casein (0.5% casein; C-7078, Sigma) by incubation at 37°C for 30 min to prevent nonspecific protein binding (Vogt et al., 1987) and again washed four times. Each sera was diluted in casein-PBS to concentrations ranging from 10^3 to 10^6 before applying 50 μL per well to triplicate wells and incubating for 60 min at 37°C. Following four washes, 100 μL per well of horseradish peroxidase-conjugated goat anti-rabbit IgG (GAR IgG-HRP; 55679, Organon Teknika Corp., West Chester, PA) diluted 1:500 in casein-PBS was applied and the plate incubated for 30 min at 37°C. Following eight washes, a substrate solution consisting of 1 mL of 300 μg/mL 2,2'-azino-bis[3-ethylbenzthiazo-line-6-sulfonic acid] (ABTS; A-1888, Sigma), 11 mL of citrate buffer (pH 4.0), and 8 μL of concentrated hydrogen peroxide was prepared and 100 μL of the solution applied to each well. Bound peroxidase activity was measured spectrophotometrically at 405 nm using a microplate reader (THERMOmax, Molecular Devices Corp., Menlo Park, CA) following 30 min of color development at ambient temperature. Titer was arbitrarily defined as the reciprocal of the highest dilution of serum to give absorbance twice that of preimmune serum at the same dilution. Serum from the second boost of rabbit #45903 was used for all further testing because of its high titer.

3.3.5 Antibody Specificity Determination by CI-ELISA. Cross-reactivity testing was done by competitive indirect (CI) ELISA to determine the protein specificity, aldehyde specificity, and amino acid specificity of the antibodies. To establish that the antibody reactivity was not solely with carrier protein, three different proteins were tested in unmodified and hexanal-modified form. In addition, free hexanal, not attached to a carrier, was tested for reactivity. Once the protein carrier requirement for recognition by the antibody was established, further testing was performed using conjugates. To characterize the "hapten-end" of the epitope, CSA conjugates of various aldehydes, ketones, or alcohols were tested for cross-reactivity. To characterize the "carrier-end" of the epitope, hexanal conjugated to various amino acids was tested. Compounds which were not conjugates, i.e. caproic acid (C-2250, Sigma), tridecanoic acid (T-0502, Sigma) and free amino acids, were diluted to a concentration of 200 mM in PBS and checked for reactivity by CI-ELISA. Cross-reactivity was defined as the protein concentration of hexanal-modified CSA required for 50% inhibition divided by protein concentration of aldehyde-modified protein for 50% inhibition (Deshpande, 1996).

The competitive assay was the same as the noncompetitive ELISA with three exceptions: 1) 50 μL per well of standard or test solution was incubated simultaneously with 50 μL per well of antiserum, 2) the antiserum dilution was 1:3160, and 3) a standard curve of hexanal-modified CSA was used. To improve the solubility of hydrophobic free hexanal, methanol in PBS at 0, 10, 20 and 30% was used for sample dilutions of hexanal and hexanal-CSA conjugate.

For the protein, aldehyde and carbonyl compound specificity experiments, conjugate concentrations are given as protein concentration, expressed in micrograms per

milliliter. For the amino acid specificity experiments, conjugate concentrations are given as amino acid concentration, expressed in millimoles per liter. For evaluation of antiserum reactivity with unmodified and hexanal-modified CSA, hexanal concentration, expressed in nanograms per milliliter, is used. The hexanal concentration of CSA-hexanal conjugate was calculated from the protein concentration based on tritium-labeling of available lysines. All experiments were run in triplicate.

3.3.6 Assay Sensitivity and Precision. To establish the working range of the CI-ELISA, a standard curve of maximally modified hexanal-CSA adduct was produced by diluting a stock solution of 15 mg/mL protein to protein concentrations of 0.01, 0.03, 0.10, 0.32, 1.0, 3.2, 10 and 32 μg/mL. Intraplate and interplate assay precisions of the CI-ELISA were determined within one microtiter plate and among eight plates, respectively, using hexanal-modified CSA solutions of 0.01, 0.1 and 1 μg/mL protein.

3.3.7 Western Blot. The antibody specificities to unmodified and hexanal-modified proteins were evaluated by the Western blot method of Wang et al. (1994). Unmodified and hexanal-modified BSA, CSA and high molecular weight standard (HMWS) protein mixture were diluted in PBS to protein concentrations of 100, 100 and 250 μg/mL, respectively. A 0.5 mL aliquot of protein solution was combined with 0.5 mL of SDS-reducing buffer (0.5M Tris-HCl, pH 6.8, 10% w/v SDS, 2-β-mercaptoethanol), and the mixture boiled for 5 min prior to loading 10 μL of each onto the gel. The resolving gel was 12% acrylamide and the stacking gel was 4% acrylamide (Laemmli, 1970). Electrophoresis was performed using a Mini-Protein Gel assembly (Bio-Rad Laboratories, Hercules, CA) run at a current of 55 mA and a voltage of 200 V for 1 hr. Transfer of the proteins from the gel to nitrocellulose membrane (BA-S 85, Schleicher and Schuell,

Keene, NH) was performed by electrophoresis for 1 hr at 350 mA and 100 V. The protein-containing membrane was washed thoroughly with PBS-Tween and blocked for 30 min at ambient temperature with PBS-casein. The membrane was washed thoroughly and incubated with anti-hexanal antiserum diluted in 0.5% PBS-casein 1:2000 for 30 min. The membrane was washed thoroughly and incubated with GAR IgG-HRP conjugate diluted in 0.5% PBS-casein 1:2000 for 10 min. The membrane was washed thoroughly, incubated with catalyzed substrate solution, and stopped after 7 min with deionized water. The substrate solution was prepared by dissolving 24 mg 3,3',5,5'-tetramethyl benzidine (TMB; T-2885, Sigma) and 80 mg dioctyl sulfosuccinate (D-0885, Sigma) in 10 mL ethanol and diluting to 40 mL with 0.1 M citrate buffer (pH 5.0). A 20 μL aliquot of 30% hydrogen peroxide was added just prior to incubation to catalyze the peroxidase-substrate reaction.

3.4 RESULTS AND DISCUSSION

3.4.1 Preparation of Hexanal Conjugates. Following reductive alkylation of CSA with hexanal, amino acid analysis of the native and hexanal-modified PITC derivatives was performed. The lysine contents were 11.6 and 0.2 mole % for native CSA and hexanal-modified CSA, respectively (Table 3.1). No change was observed in the content of any other amino acid. Similarly, Weisgraber et al. (1978) subjected LDL to reductive methylation using formaldehyde and compared the amino acid compositions of the native and modified LDL solutions. They reported modification of 18 of 20 lysine residues and no changes in any other residues. These findings were expected since, the reducing agent,

Table 3.1 Amino acid composition (mole percent) of native and hexanal-modified chicken serum albumin (CSA)

Amino Acid	Native CSA	Modified CSA
ASx	6.3	9.4
GLx	13.1	13.9
SER	6.4	7.2
GLY	5.6	5.1
HIS	2.0	2.7
ARG	4.7	5.5
THR	3.6	4.1
ALA	8.0	8.7
PRO	7.0	7.3
TYR	3.6	4.2
VAL	6.3	7.0
MET	2.1	4.0
ILE	6.0	6.3
LEU	8.0	8.4
PHE	5.7	6.3
LYS	11.6	0.2

$NaCNBH_3$, is highly specific and reduces only Schiff bases formed via ε-amino groups of lysine residues and α-amino termini and not aldehydes (Jentoft and Dearborn, 1979).

The amount of tritium incorporated into CSA during protein labeling via reductive alkylation was 32.6 moles per mole CSA. Stoichiometrically, this corresponds to 32.6 moles of lysine or hexanal per mole CSA. Using the molar ratio of 32:1 hexanal to CSA, the hexanal concentration of CSA-hexanal conjugate was calculated from the protein concentration. The calculation, given molecular masses of 66,300 and 100 daltons for CSA and hexanal, respectively, was as follows:

$$\text{hexanal } \frac{ng}{mL} = \frac{\mu g}{mL}\ \text{CSA} \times \frac{[1\ \mu\text{mole CSA}]}{[66{,}300\ \mu g\ \text{CSA}]} \times \frac{[32\ \mu\text{moles hexanal}]}{[1\ \mu\text{mole CSA}]} \times \frac{[100\ \mu g\ \text{hexanal}]}{[1\ \mu\text{mole hexanal}]} \times \frac{[1000\ ng]}{[1\ \mu g]}$$

Since the values determined by tritium-labeling concurred with values of 28-31 moles of modified lysine per mole of CSA as determined by TNBS assay, the TNBS assay was used in all subsequent experiments to determine modification. The degrees of modification for the hexanal-BSA conjugates used in antibody production were 97.2%, 97.1%, 97.6%, and 98.4% for the primary injection and first, second, and third boosts, respectively, as determined by TNBS assay.

3.4.2 Antiserum Titers. Polyclonal antisera which recognize protein-bound hexanal were successfully produced in rabbits. The highest titers achieved, 6.15×10^{-5} and 6.28×10^{-5}, were from the antisera of rabbits 45903 and 45904 following the second boost (Table 3.2). Rabbit 45911 produced its highest serum titer, 1.64×10^{-5}, following the third boost. Palinski et al. (1990) reported titers of greater than 10^{-5} for polyclonal antisera to MA-modified LDL after a primary and two booster injections. The antiserum collected

Table 3.2 Serum titers of polyclonal antibodies against hexanal-modified bovine serum albumin (BSA) in rabbits[1]

Week[2]	Titer x 10^{-5}		
	Rabbit 45903	Rabbit 45904	Rabbit 45911
5	2.38	3.07	0.30
9	6.15	6.28	0.49
19	1.00	0.90	1.64
76	1.09	0.28	--

[1] The titer of each serum was arbitrarily designated as the maximum dilution that yielded twice the absorbance of the same dilution of preimmune serum in an indirect enzyme-linked immunosorbent assay.

[2] Subcutaneous booster injections were performed at weeks 4, 8, 18 and 75 using 500 μg of hexanal-modified BSA

following the second boost of rabbit 45903 was used for all subsequent testing since it had one of the highest titers.

3.4.3 Assay Sensitivity and Precision. The assay sensitivity, or limit of detection, defined as two times the standard deviation of the mean response of the zero standard (Deshpande, 1996), was determined to be 1.5 nanograms hexanal per milliliter using hexanal-modified CSA in the CI-ELISA. The working range, or linear portion, of the assay for hexanal-modified CSA was 0.03-3 μg/mL protein. A standard curve of hexanal concentration versus absorbance was generated by substituting hexanal concentration for protein concentration based on the amount of hexanal bound during reductive alkylation as determined by tritium incorporation (Figure 3.1). The intraplate coefficient of variation (CV) values for hexanal-CSA adduct were 4.0, 8.4 and 13.9% (n = 21) for protein concentrations of 0.01, 0.1 and 1 μg/mL, respectively. The interplate CV values for hexanal-CSA adduct among 8 plates were 6.6% (n = 94), 8.4% (n = 93) and 13.0% (n = 94) for protein concentrations of 0.01, 0.1 and 1 μg/mL, respectively.

3.4.4 Protein Specificity. CSA, KLH and BSA were substantially modified by reductive alkylation with hexanal as indicated by loss of reactive amino groups as measured by TNBS assay. The degrees of modification of hexanal-modified CSA, KLH, and BSA were 95.3%, 86.8%, and 94.8%, respectively. By competitive ELISA, the antiserum did not show recognition of the unmodified forms of CSA, KLH or BSA, yet did bind to each of the hexanal-modified proteins (Figure 3.2). Lack of recognition of unmodified BSA, the immunization carrier protein, suggests that the protein was modified sufficiently to present primarily hexanal moieties as the antigenic determinants. Hexanal-modified KLH required a concentration of approximately 30 times that of modified CSA or modified

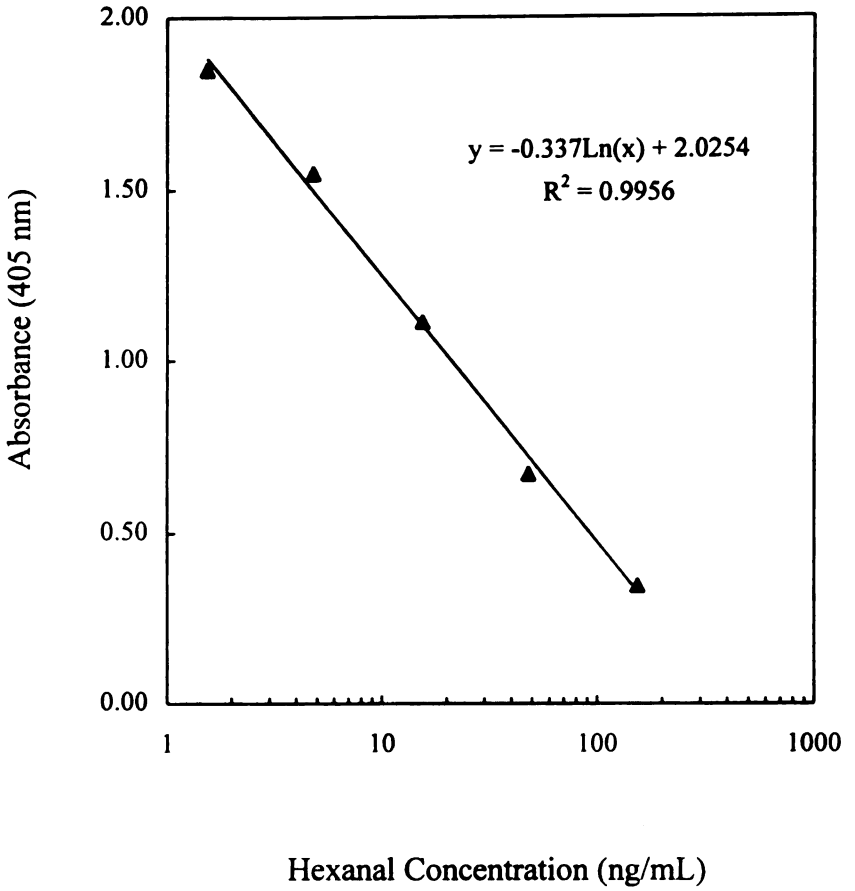


Figure 3.1 Standard curve of hexanal-modified chicken serum albumin (CSA). Hexanal concentration determined from protein concentration, based on amount of tritium incorporated into hexanal-CSA during reductive alkylation.

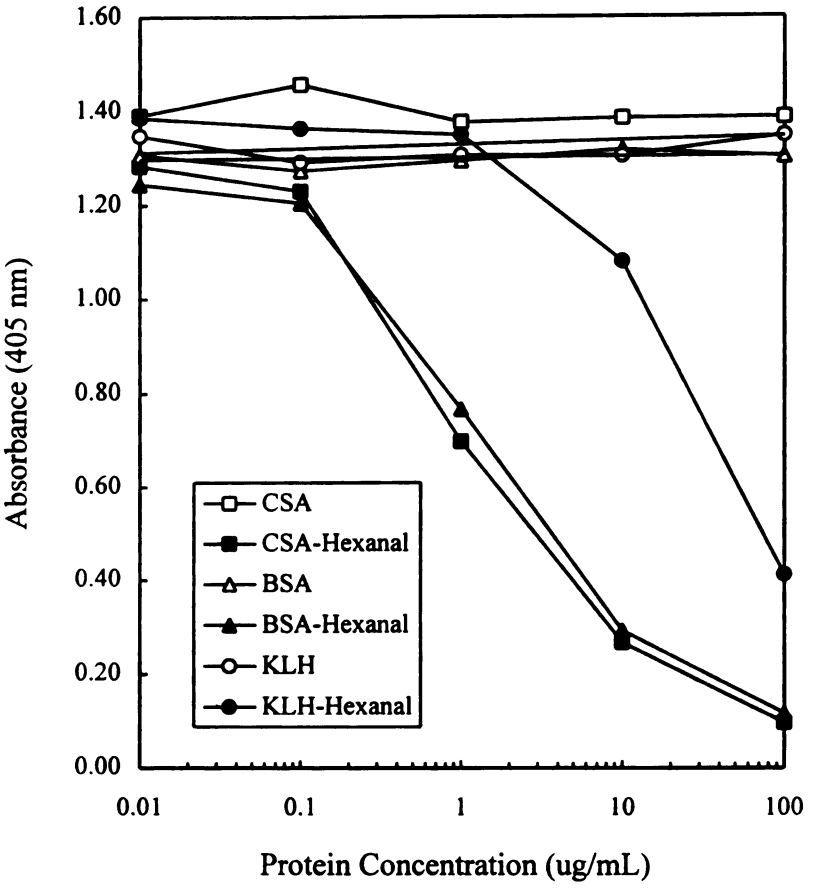


Figure 3.2 Protein specificity of antibodies to hexanal-modified bovine serum albumin (BSA) was determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified chicken serum albumin (CSA) and incubated with antiserum diluted 1:3160. Unmodified and modified CSA, BSA and keyhole limpet hemocyanin (KLH) competed at concentrations of 0.01, 0.1, 1, 10 and 100 μg/mL. Determinations were performed in triplicate.

BSA to achieve 50% binding inhibition, likely due in part to the lower degree of amino group modification and in part to fewer lysine derivatives per mole protein.

A Western blot of unmodified and hexanal-modified BSA, CSA and a HMWS protein mixture showed antiserum recognition of the hexanal-modified proteins but not the unmodified forms (Figure 3.3), confirming the results of the ELISA. Hexanal-modified proteins reacted with antiserum regardless of molecular weight. For the modified HMWS, bands are visible for myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97.4 kD), BSA (66 kD) and OA (45 kD) suggesting the immuno-dominant epitope is a structure common to all hexanal-modified proteins.

Free hexanal, at concentrations of 7, 70, and 700 ng/mL, did not inhibit antibody binding regardless of the methanol concentration (Figure 3.4). Methanol at concentrations of 0, 10, 20 and 30% was added to improve the solubility of unbound hexanal since hexanal-protein conjugate in 10, 20 and 30% methanol showed little difference in binding from the control (0% methanol). While methanol did not appear to interfere with the assay, it did not increase free hexanal reactivity. Since antibodies can only be made to haptens by first coupling them to a protein carrier, in many cases the antibody elicited will not react with the free, native molecule, but only with the conjugated version (Morris, 1995). Our finding suggests the antiserum recognizes an epitope larger that the six-carbon hexanal molecule.

3.4.5 Aldehyde Specificity. CSA was modified by reductive alkylation with various aldehydes (Table 3.3). Modification, expressed as percent loss in reactive amino groups, was at least 90.8%. This finding eliminates the possibility that differences in epitope density caused by varying degrees of modification are responsible for differences in

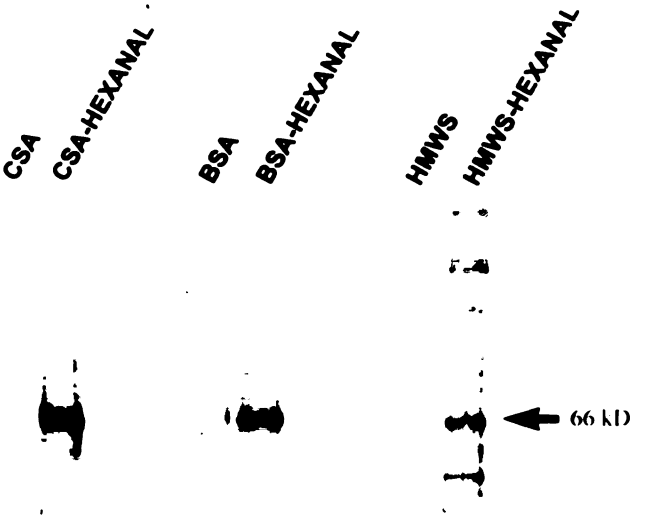


Figure 3.3 Western blot of unmodified and hexanal-modified chicken serum albumin (CSA; Lanes 1 and 2), bovine serum albumin (BSA; Lanes 3 and 4) and high molecular weight standard (HMWS; Lanes 5 and 6) containing myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97.4 kD), BSA (66 kD) and ovalbumin (45 kD).

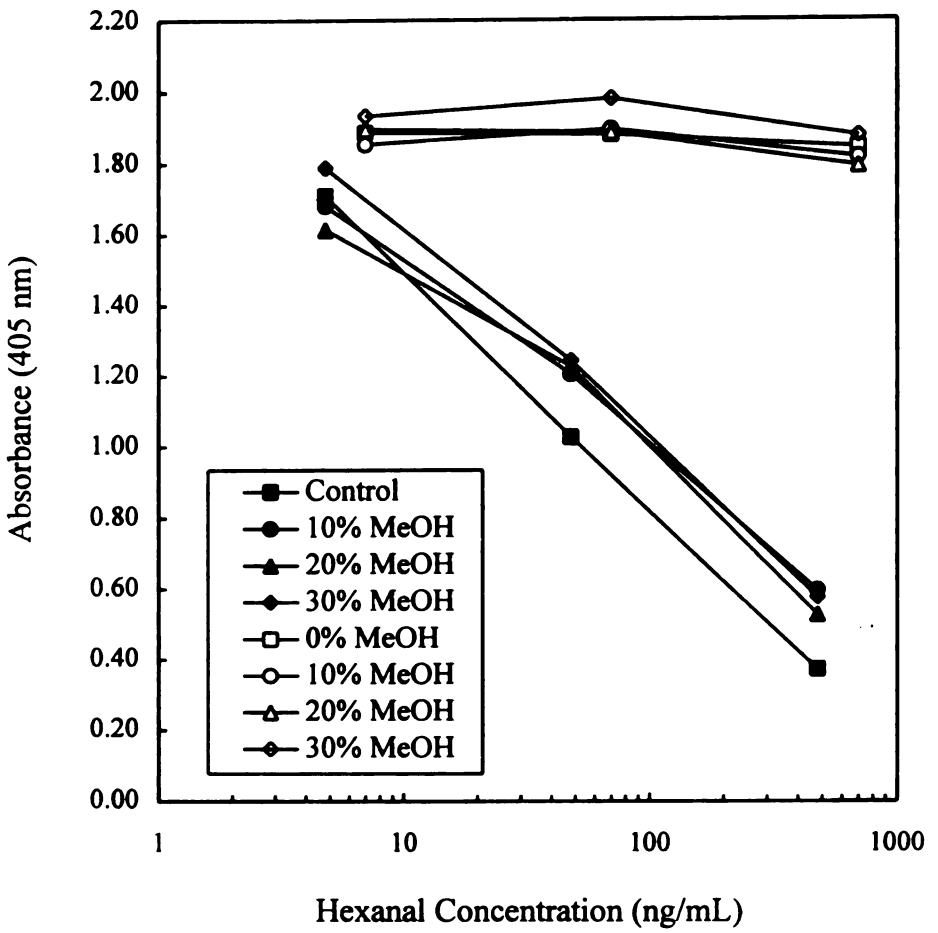


Figure 3.4 Specificity of antibodies to hexanal-modified bovine serum albumin was determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified chicken serum albumin (CSA) and incubated with antiserum diluted 1:3160. Free hexanal (open symbols) or CSA-conjugated hexanal (closed symbols) competed in 0, 10, 20 or 30% methanol. Hexanal concentrations of conjugates were based on tritium labeling results. Determinations were performed in triplicate.

Table 3.3 Concentration of reactive amino groups in native and modified chicken serum albumin (CSA) solutions as measured by trinitrobenzene sulfonic acid (TNBS) assay

Solution	Reactive amino groups (mole NH_2/mole CSA)	Loss[1] (%)
Native CSA	31.0	--
Propanal-CSA	2.2	92.9
Butanal-CSA	2.3	92.5
Pentanal-CSA	2.1	93.1
Hexanal-CSA	2.3	92.6
Heptanal-CSA	2.0	93.4
Octanal-CSA	2.3	92.7
Nonanal-CSA	2.9	90.8
2-*t*-hexenal-CSA	0.6	98.1
2-Methylpentanal	0.9	97.0
2-Methylbutanal	0.9	97.0
3-Methylpentanal	1.2	96.2

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

binding inhibition among the conjugates. Antiserum cross-reactivity occurred to some extent with all of the aliphatic aldehydes tested, except propanal, and appeared to increase as the number of carbons approached six (Figure 3.5). Heptanal-modified CSA showed the highest cross-reactivity at 86.3% followed by pentanal- and octanal-modified CSA at 11.8% and 1.9%, respectively (Table 3.4). The differences in antiserum reactivity suggest that antibodies can discriminate between differences in the carbon number of the aldehyde. Previous reports support the same conclusion. Klassen et al. (1990) reported that antiserum to acetaldehyde-modified tubulin showed decreasing recognition of aldehyde-modified BSA with increasing aldehyde carbon number with relative absorbances of 1.000, 0.802, and 0.531 for acetaldehyde (2C), propanal (3C), and butanal (4C) adducts, respectively. Perata et al. (1991) reported that antiserum to acetaldehyde-modified KLH exhibited the following cross-reactivities with aldehyde-modified BSA: 6.6, 100, 106, 0, and 0% for formaldehyde, acetaldehyde, propanal, butanal, and pentanal, respectively. In another study, an antiserum to carbamylated-LDL was produced (Steinbrecher et al., 1984) which recognized homocitrulline (ε-carbamyl-diaminocaproic acid), a lysine derivative. Homocitrulline was highly competitive with carbamylated-LDL while citrulline (δ-carbamyl-diaminopentanoic acid) containing one fewer carbon was a very weak competitor. Cross-reactivity of our antibodies with heptanal and pentanal should not compromise the usefulness of an ELISA for monitoring lipid oxidation for two reasons. First, the concentrations of either heptanal and pentanal in chicken may be 30 times less than that of hexanal in oxidized lipid-containing samples (Ajuyah et al., 1993). Second, both aldehydes are lipid oxidation products, so cross-reactivity with these aldehydes will not make the assay nonspecific for lipid oxidation.

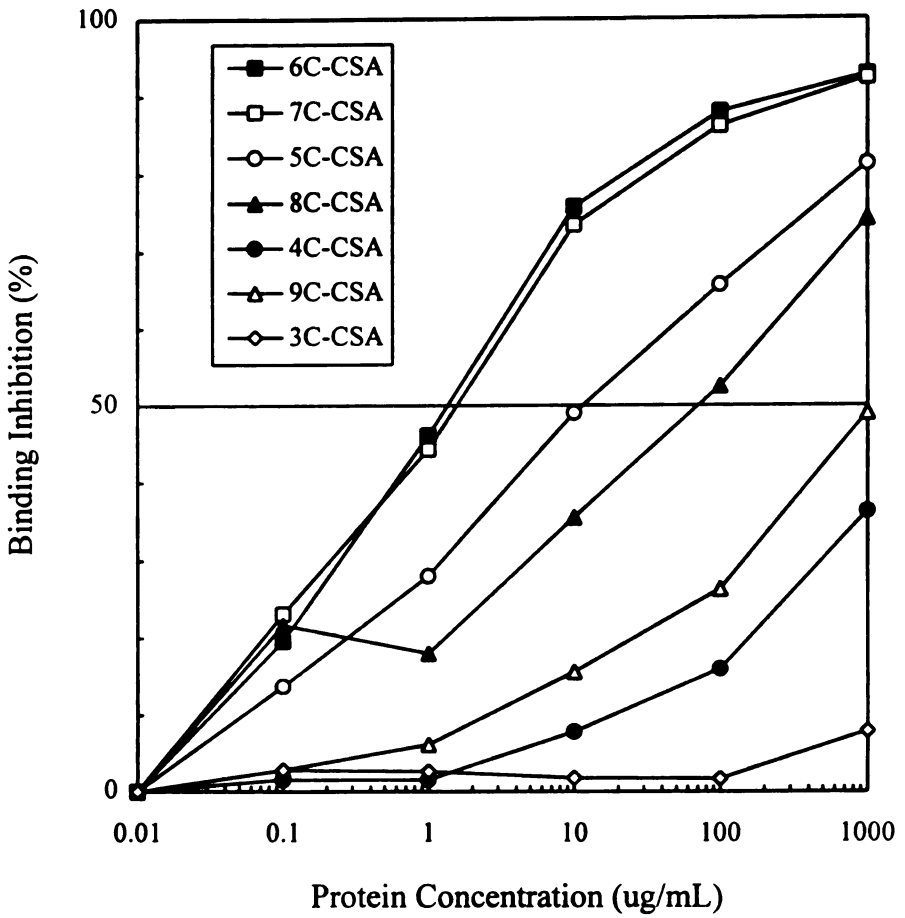


Figure 3.5 Aliphatic aldehyde specificity of antibodies to hexanal-modified bovine serum albumin was determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified chicken serum albumin (CSA) and incubated with antiserum diluted 1:3160. Aldehyde-modified CSA competed at protein concentrations of 0.01, 0.1, 1, 10, 100 and 1,000 μg/mL. Aldehydes were: propanal (3C), butanal (4C), pentanal (5C), hexanal (6C), heptanal (7C), octanal (8C) and nonanal (9C). Determinations were performed in triplicate.

Table 3.4 Cross-reactivity of polyclonal antibodies against hexanal-modified bovine serum albumin with aldehyde-modified chicken serum albumin (CSA) by indirect competitive ELISA

Aldehyde Component	Chemical Structure	Cross-Reactivity[1] (%)
Hexanal	CSA-$NH(CH_2)_5CH_3$	100.0
2-*t*-Hexenal	CSA-$NHCHCH{=}CH(CH_2)_2CH_3$	100.0
Heptanal	CSA-$NH(CH_2)_6CH_3$	86.3
Pentanal	CSA-$NH(CH_2)_4\ CH_3$	11.8
3-Methylbutanal	CSA-$NH(CH_2)_2CH(CH_3)CH_3$	2.2
Octanal	CSA-$NH(CH_2)_7CH_3$	1.9
2-Methylpentanal	CSA-$NHCH_2CH(CH_3)(CH_2)_2CH_3$	1.1
Butanal	CSA-$NH(CH_2)_3CH_3$	0.3
Nonanal	CSA-$NH(CH_2)_8CH_3$	0.1
Propanal	CSA-$NH(CH_2)_2CH_3$	<0.1
2-Methylbutanal	CSA-$NHCH_2CH(CH_3)CH_2CH_3$	<0.1

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

The competitive abilities of other carbonyl compounds were considered as well. CSA conjugates of the Strecker degradation products, 2-methylbutanal, 2-methylpentanal, and 3-methylbutanal, were evaluated for cross-reactivity. These branched aldehyde conjugates were considerably less effective competitors than the aliphatic ones (Figure 3.6). The cross-reactivities of 3-methylbutanal- and 2-methylpentanal-modified CSA were 2.2% and 1.1%, respectively (Table 3.4). In addition, conjugates of the ketone, 2-heptanone, and the alcohols, n- and 2-hexanol, were evaluated. CSA modified with 2-heptanone proved to be a weak competitor, in part, due to modification of only 83.6% as measured by TNBS assay. The alcohols were unreactive with the antibodies, likely due to their lack of a carbonyl group preventing reaction with CSA during reductive alkylation. The monounsaturated six-carbon aldehyde, 2-*trans*-hexenal, showed cross-reactivity of 100%, likely due its length and to reduction of its double bond during reductive alkylation.

3.4.6 Amino Acid Specificity. Hexanal conjugates of the amino acids, glycine, lysine (α-,ε-diaminocaproic acid) and arginine, and the lysine derivative, ε-aminocaproic acid (ACA), were evaluated for competitiveness by CI-ELISA. In addition, γ-aminobutanoic acid (GABA) was modified with octanal to achieve a molecule of length equal to that of hexanal-modified ε-ACA. The difference in structures was in the position of the nitrogen atom (Figure 3.7). While neither the free amino acids nor free ε-ACA were not recognized by the antiserum, hexanal-modified ε-ACA was a highly effective competitor and, in fact, a better competitor than hexanal-modified lysine (Figure 3.8). The concentration required for 50% antibody binding inhibition was 1091 mM for lysine-hexanal conjugate compared to 234 mM for ε-ACA-hexanal conjugate (Table 3.5). Palinski et al. (1989) reported that MA-modified ε-ACA and MA-modified t-

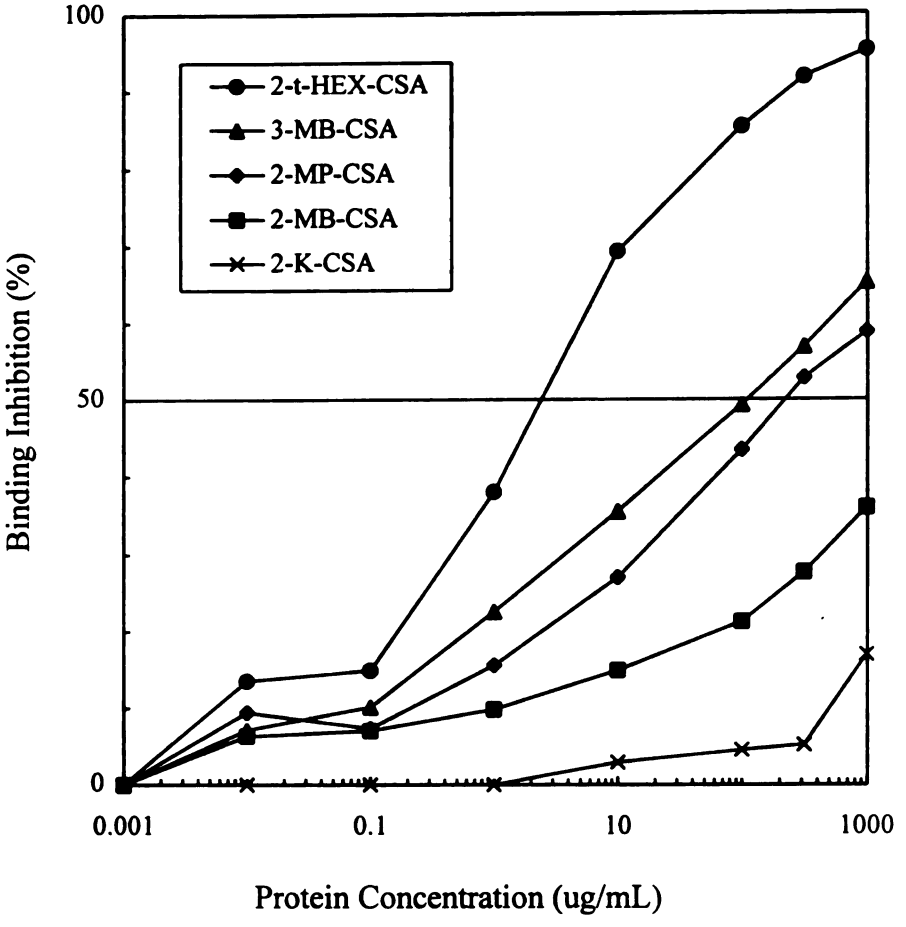
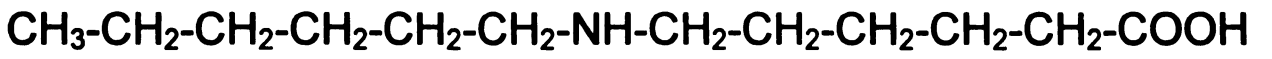
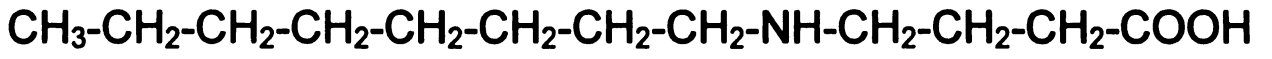


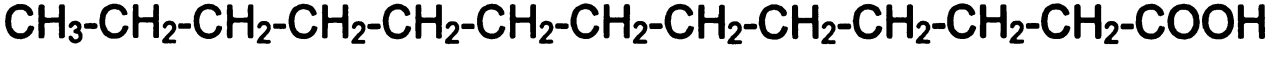
Figure 3.6 Carbonyl compound specificity of antibodies to hexanal-modified bovine serum albumin was determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified chicken serum albumin (CSA) and incubated with antiserum diluted 1:3160. Modified CSA competed at protein concentrations of 0.001, 0.01, 0.1, 1, 10, 100 and 1,000 μg/mL. Modifying compounds were: 2-*trans*-hexenal (2-t-HEX-CSA), 3-methylbutanal (3-MB-CSA), 2-methylpentanal (2-MP-CSA), 2-methylbutanal (2-MB-CSA) and 2-hexanone (2-K-CSA). Determinations performed in triplicate.



Hexanal-modified epsilon-aminocaproic acid



Octanal-modified gamma-aminobutanoic acid



n-tridecanoic acid

Figure 3.7 Structures of thirteen atom compounds: hexanal-modified ε-aminocaproic acid, octanal-modified γ-aminobutanoic acid and tridecanoic acid.

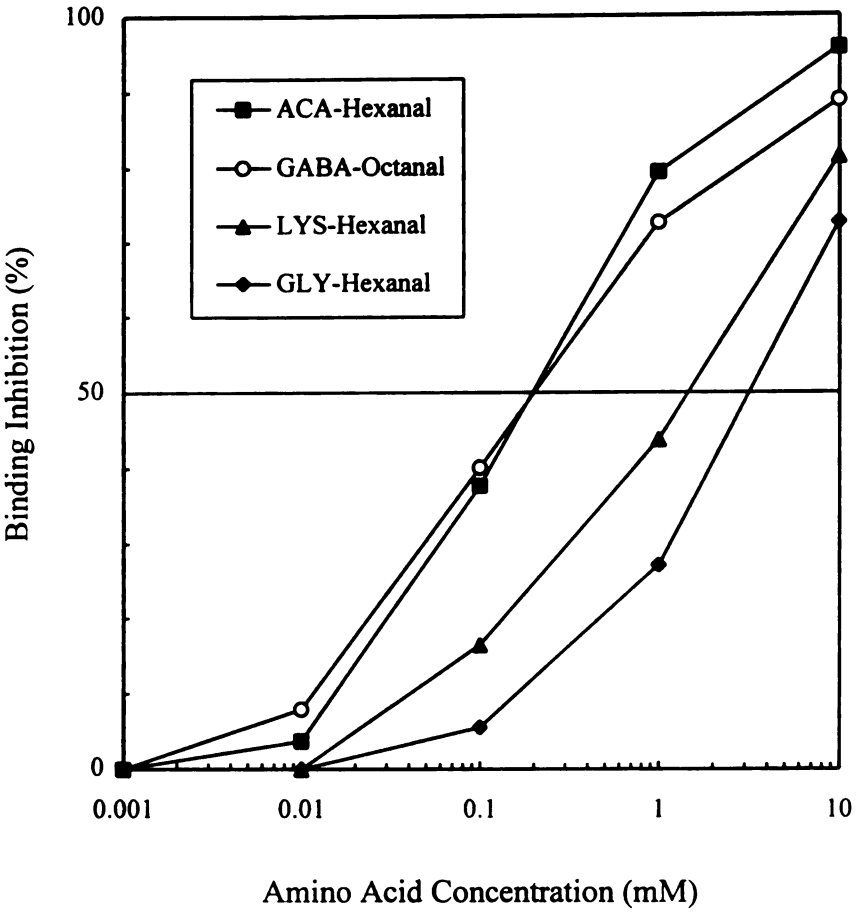


Figure 3.8 Acid specificity of antibodies to hexanal-modified bovine serum albumin was determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified chicken serum albumin and incubated with antiserum diluted 1:3160. Conjugates competed at concentrations of 0.001, 0.01, 0.1, 1 and 10 mM. Conjugates were: ε-aminocaproic acid (ACA)-hexanal, γ-aminobutanoic acid (GABA)-octanal, lysine (LYS)-hexanal, glycine (GLY)-hexanal. Determinations performed in triplicate.

Table 3.5 **Reactivity of polyclonal antibodies against hexanal-modified bovine serum albumin with aldehyde-amino acid conjugates by competitive indirect ELISA**

Conjugate	Concentration Required for 50% Inhibition (mM)
ε-Aminocaproic acid-Hexanal	234
γ-Aminobutanoic acid-Octanal	260
Lysine-Hexanal	1090
Glycine-Hexanal	3090

butoxycarbonyllysine could compete, binding antibodies to MA-modified LDL. The lower reactivity of modified lysine may be due to steric hindrance caused by the proximity of the α- and ε-amino groups. Hexanal-modified glycine and arginine were considerably less competitive. Their reactivity is likely attributable to modification of the α-amino groups which were not blocked. Jentoft and Dearborn (1979) used nine N^{α}-acetyl amino acids to determine which amino acids had side chains capable of forming stable derivatives with formaldehyde under reductive methylation conditions and concluded that arginine, histidine, methionine and tryptophan did not. They found that tyrosine, serine, asparagine and cysteine could form stable derivatives but at a reaction rate at least 1000-fold slower than N^{α}-acetyllysine.

Our experimental findings indicate that our antiserum recognizes protein-bound hexanal regardless of the protein carrier and can discriminate differences as small as one carbon in the aldehyde chain-length. We propose that the immunodominant epitope is a hexanal-modified lysine residue. Tuma et al. (1987) examined the effect of reaction conditions on the types and relative amounts of products formed between acetaldehyde and BSA. When sodium cyanoborohydride was used, the predominant product was the acetaldehyde-derivatized lysine, *N*-ethyllysine. Two additional compounds, formed in much higher amounts when a reducing agent was not used, were found in minimal quantities when cyanoborohydride was used. While the molecular structures of these two adducts were not established, they were determined to be modified lysine residues. Our results suggest that the modification to the lysine residues occurring during reductive alkylation is the addition of a hexanal molecule producing a hexanal-modified lysine residue. While the length of the epitope (number of atoms) seems to significantly

influence the ability of the antibodies to recognize the structure, the presence of a nitrogen atom may also be important. In a comparison of hexanal-modified ε-ACA and octanal-modified GABA, structures with the same chain length but with the nitrogen atom at a different position, the two compounds compete equally well (Figure 3.8). However, the 13-carbon carboxylic acid, tridecanoic acid, was not reactive suggesting the requirement of a nitrogen atom for reactivity with these antibodies.

The results of this study demonstrate that sensitive and specific polyclonal antibodies that react primarily to hexanal-modified lysine adducts can be produced. Development of an ELISA incorporating these antibodies to monitor lipid oxidation in biological systems by measuring the hexanal content, therefore, seems feasible.

CHAPTER 4

ELISA TO MONITOR LIPID OXIDATION IN A MEAT MODEL SYSTEM BY MEASUREMENT OF HEXANAL CONTENT

4.1 ABSTRACT

Lipid oxidation in fresh, frozen or cooked meat is generally associated with the development of objectionable flavors and odors and, therefore, can be responsible for limiting shelf-life or loss of consumer acceptability. Hexanal content has been used as an indicator of the extent of lipid oxidation in meats and other foods. The purpose of this study was to demonstrate the potential use of a hexanal-specific antibody-based ELISA to measure hexanal concentration in muscle, by correlating ELISA results to results determined by gas chromatography.

Two competitive indirect (CI) ELISAs were designed using anti-hexanal-BSA antibodies: one using polyclonal antibodies, developed in a previous study, and one using a monoclonal antibody, developed in a separate study. First, each assay was tested for its ability to detect differences in the hexanal content of solutions of ovalbumin and chicken muscle homogenate spiked with hexanal to different concentrations. Second, both assays were used to monitor lipid oxidation in a meat model system by measuring the hexanal content of chicken thigh homogenate subjected to an accelerated oxidation at 50°C for 36 hr. Because the antibodies were shown in previous studies to recognize hexanal-lysine

adducts and not free hexanal, a reductive alkylation procedure was incorporated into sample preparation prior to testing by ELISA. Third, the hexanal contents in the meat model system as determined by each ELISA were compared with the results of a dynamic headspace gas chromatography (HS-GC) method using Tenax traps and a thiobarbituric acid-reactive substances (TBA-RS) assay. The polyclonal antibody-based CI-ELISA showed strong correlations with both the HS-GC and TBA-RS methods with coefficients of 0.89 and 0.85, respectively. The monoclonal-based ELISA gave similar results. The results of our study indicate that use of our ELISAs to monitor lipid oxidation in chicken thigh homogenate is a faster and simpler alternative to GC.

4.2 INTRODUCTION

Lipid oxidation occurring in fresh, frozen or cooked meat is generally associated with the development of rancid flavors and odors and a concomitant reduction in the acceptability of meat (Gray et al., 1994). Therefore, the potential for lipid oxidation and the development of rancid flavor is one of the single greatest constraints in determining the processing and shelf-life characteristics of muscle foods (Allen and Foegeding, 1981). While hydroperoxides, the primary products of lipid oxidation, are odorless and tasteless, their degradation leads to the formation of complex mixtures of low-molecular weight compounds with distinctive odor and flavor characteristics (Shahidi and Pegg, 1994). The flavor significance of a particular volatile compound depends on the combination of its individual threshold value and its concentration in the sample (Gray et al., 1994). Aliphatic breakdown products are significant to meat flavor due to their low odor threshold values and increasing concentrations during oxidation (Shahidi and Pegg, 1994).

One of the most important precursors of aldehyde compounds is linoleic acid due to its high susceptibility to oxidation, abundance in foods (Kochhar, 1993) and ubiquity in muscle tissue (Shahidi, 1994). Hexanal is the only aldehyde to autoxidatively arise from both linoleic acid hydroperoxides and some of the unsaturated aldehyde products (Schieberle and Grosch, 1981). Hexanal has an odor threshold of 5 ng/mL in water (Buttery et al., 1988) and 6 μg/mL in cooked, lean ground beef (Brewer and Vega, 1995).

The ultimate criterion for suitability of any compound as an index of lipid oxidation is its adequate correlation with sensory data (Shahidi, 1994). Hexanal content has been shown to correlate well with off-flavor development during storage of cooked ground beef (St. Angelo et al., 1987), cooked ground pork (Shahidi et al., 1987), cooked chicken breast and thigh (Ang and Lyon, 1990) with coefficients of 0.80, 0.98, 0.92 and 0.84, respectively. Hexanal has been used successfully to follow lipid oxidation in meat products (Gray et al., 1994); however, the bulk of the reported studies evaluated cooked meats. Hexanal concentrations may also be used for evaluating frozen raw and cured-meat products where oxidation proceeds slowly (Shahidi and Pegg, 1994). Stoick et al. (1991) compared changes in hexanal concentrations of restructured beef steaks during 6 months of frozen storage to evaluate the antioxidant efficacy of several different substances. Hexanal was one of several volatile compounds used to compare the extent of lipid oxidation among frozen ground pork packaged in different materials (Brewer et al.,1992) and between fresh farmed-raised versus wild alligator tail meat (Cadwalladar et al., 1994). Analysis of the volatile compounds of fresh irradiated chicken showed increased hexanal content with increased radiation dose (Hansen et al., 1987). Any degree of oxidation occurring in raw tissue can accelerate the development of oxidized off-flavors

in cooked products due to the free radical chain reaction mechanism of autoxidation (Rhee, 1988).

Hexanal content has potential for use as an indicator for quality control purposes during processing and storage of meat products (Shahidi and Pegg, 1994). Unfortunately, static headspace techniques, while simple, rapid and reproducible, often lack sensitivity since insufficient quantities of indicator compounds are obtained (Reineccius, 1996). Improved sensitivity is achieved by use of dynamic, or purge-and-trap, techniques which use an adsorbent material, such as Tenax, to collect volatiles over a period of time but are, therefore, not rapid methods. As an analytical tool, the immunoassay has already proven to be very useful for the assessment of a variety of substances in meat products, including various meat species, non-meat proteins, pesticides, and microorganisms (Fukal, 1991). Immunoassays are a rapid, cost-effective, and easy-to-use alternative to conventional analytical techniques achieving sensitivity and specificity without requiring highly trained analysts or sophisticated equipment (Samarajeewa et al., 1991). In addition, immunoassay kits are portable and can be used for routine surveillance in a processing facility or retail outlet.

This study was undertaken to develop a novel immunoassay for monitoring lipid oxidation in meat and other foods and, in particular, to replace more labor-intensive and time-consuming headspace gas chromatography (HS-GC) methods. We reported the production and characterization of polyclonal (Chapter 3) and monoclonal antibodies (Zielinski, 1997) to hexanal-modified bovine serum albumin (BSA). Both were found to be sensitive and specific for protein-bound hexanal independent of the carrier. This study consisted of three specific objectives. The first objective was to show that both the

polyclonal-based and monoclonal-based assays could detect differences in the hexanal content of solutions of ovalbumin and chicken muscle homogenate spiked with hexanal to different concentrations. The second objective was to monitor lipid oxidation in a meat model system using both ELISAs to measure the hexanal content. Because the antibodies recognize hexanal-lysine adducts and not free hexanal, a reductive alkylation procedure was incorporated into sample preparation prior to testing by ELISA. Sample preparation of muscle tissue, as part of the meat model system, consisted of three steps: 1) tissue homogenization and protein extraction, 2) accelerated oxidation, and 3) reductive alkylation. The third objective of this study was to compare the hexanal contents in the meat model system determined by each ELISA with the results of a dynamic headspace gas chromatography (HS-GC) method using Tenax traps and a thiobarbituric acid-reactive substances (TBA-RS) assay.

4.3 MATERIAL AND METHODS

4.3.1 Preparation of Muscle Homogenate. The first step in the preparation of muscle tissue samples was to produce a protein extract homogenate as part of a meat model system. Homogenates were prepared from chicken thigh muscle and from boneless, skinless chicken breast purchased from a local retail store. For the thigh muscle homogenate, the muscle was deboned and frozen at -80°C with skin and fat retained. The frozen muscle was shattered with a hammer to pieces of a 2 cm diameter or less and combined with equally sized pieces of dry ice in a Waring blender (Model 1120, Winsted, CT). The pieces were pulverized to a fine powder with 30 sec of mixing on high speed. Three volumes of 0.3 M sodium chloride solution were added to extract both myofibrillar

and sacroplasmic proteins. The solution was mixed at high speed for 60 sec and strained through 2 layers of cheese cloth. For the preparation of breast muscle homogenate, the muscle was trimmed of visible fat and connective tissue and ground twice through a Hobart Kitchen-Aid food grinder (Model KF-A, Troy, OH) using a 4 mm plate. The ground muscle was combined with three volumes of 0.3 M sodium chloride solution, mixed and strained as described above.

4.3.2 Hexanal Modification of Protein Solutions. Solutions of chicken serum albumin (CSA; A-3014, Sigma), ovalbumin (OA, chicken egg albumin; A-5253, Sigma) and chicken muscle homogenate were modified by reductive alkylation with excess hexanal to achieve maximal modification. Maximal modification was defined as greater than 90% loss of reactive amino groups from the carrier protein. Alkylation of protein was performed by the method of Steinbrecher et al. (1984) with modifications described in Chapter 3. Briefly, hexanal (H-9008, Sigma Chemical Co., St. Louis, MO) was added to the carrier protein diluted in phosphate-buffered saline (PBS; 0.1M sodium chloride, 0.01M sodium phosphate, pH 7.4) and the solution vortexed. Reducing conditions were created by adding 1 part 800 mM sodium cyanoborohydride (NaCNBH; S-8628, Sigma) in 0.1N NaOH to 9 parts of the hexanal-protein mixture. The resultant reaction concentrations were: 200 mM hexanal, 15 mg/mL protein, and 80 mM cyanoborohydride at pH 8.5-9.0. Each solution was vortexed, held for 12 hr and dialyzed overnight at ambient temperature against two changes of PBS.

OA and chicken thigh homogenate were also differentially modified with hexanal as described above with the following exceptions. To prepare differentially-modified proteins, aliquots of 1.23, 2.46, 4.92, 7.38, 9.84, 12.3, 18.5, 24.6, 30.8 and 246 μL of

hexanal were added to solutions of 10 mg/mL protein to yield solutions of 1, 2, 4, 6, 8, 10, 15, 20, 25 and 200 mM hexanal and alkylation performed as described above. The hexanal concentrations were those prior to dialysis since unbound hexanal would have been lost in the dialysate. The degree of modification of each protein was calculated as a percent loss based on reactive amino group concentrations before and after reductive alkylation, as determined by trinitrobenzenesulfonic acid (TNBS) assay (Habeeb, 1966).

Prior to analysis by ELISA, the maximally-modified solutions of CSA, OA and chicken breast muscle homogenate were diluted to achieve protein concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μg/mL. The differentially-modified solutions of OA and chicken thigh muscle homogenate were diluted 1:500 to a protein concentration of 20 μg/mL. Each sample set was subjected to CI-ELISA as described in Chapter 3 and the results were calculated as micrograms hexanal per gram of meat based on a standard curve of hexanal-modified CSA. All determinations were made in triplicate.

4.3.3 Accelerated Lipid Oxidation Model System. The second step in the sample preparation of muscle, as part of the meat model system, was to combine 4.8 mL chicken thigh muscle homogenate with 16.8 mL of PBS in 50 mL polypropylene centrifuge tubes. Lipid oxidation of chicken thigh homogenate was induced by heating in a 50°C water bath over a 36 hr period. Sets of three solutions were immersed in the water bath every 6 hr and held until the first set had heated 36 hr. The last set, time 0, was not heated. Aliquots of homogenate from each solution were withdrawn for analysis by TBA-RS assay and by ELISA following reductive alkylation. The remaining solutions were frozen to -20°C in the tubes in which they were heated until prepared for analysis by gas chromatography.

4.3.4 Hexanal Measurement by CI-ELISA. The third and final step of sample preparation, as part of the meat model system, was reductive alkylation. One milliliter of the oxidized thigh muscle homogenate solution was combined with 1 mL of 160 mM cyanoborohydride to reduce covalent bonds formed between the muscle proteins and carbonyl compounds produced during oxidation of the chicken lipids and phospholipids. Incubation and 6 hr of dialysis were performed as described previously.

The protein-bound hexanal concentration of each reduced sample was determined by CI-ELISA. In addition to the polyclonal-based assay, a monoclonal antibody-based assay was also used. The monoclonal-based CI-ELISA incorporated supernatant from a hybridoma, designated 45P1-D9, produced by Zielinski (1997) from mice immunized with hexanal-modified BSA. The monoclonal-based assay was performed in a manner identical to that of the polyclonal assay except that hybridoma supernatant diluted 1:500 was substituted for polyclonal antiserum diluted 1:3160 and goat-anti-mouse (GAM)-horseradish peroxidase (HRP) was used instead of goat-anti-rabbit (GAR)-HRP. Determinations were made in triplicate. The intraplate precision of the polyclonal-based assay was determined using hexanal-chicken muscle protein adduct at protein concentrations of 0.1, 1 and 10 μg/mL.

4.3.5 TBA-RS Assay. The method of Buege and Aust (1978) was used to quantify thiobarbituric acid reactive substances (TBA-RS) in the lipid oxidation model system. Following accelerated oxidation, a 2 mL aliquot of homogenate was transferred to a 15 mL centrifuge tube containing 4 mL of TBA reagent. The composition of the TBA reagent was 15% w/v TBA (T-5500, Sigma), 0.375% w/v trichloroacetic acid (TCA; 0414-01, J.T. Baker, Inc, Phillipsburg, NJ) and 0.25 N hydrochloric acid. The samples

were capped loosely, boiled for 15 min, and centrifuged at 1000 x g for 15 min at ambient temperature. The absorbance of the supernatant of each solution was determined spectrophotometrically at 535 nm. The results were calculated as milligrams malonaldehyde per kilogram of meat using a molar extinction coefficient of 1.56×10^{-5} L x $mole^{-1}$ x cm^{-1}. Determinations were made in triplicate.

4.3.6 Isolation and Concentration of Volatile Compounds. Dynamic headspace gas chromatography (HS-GC) methods of Liu et al. (1992) and Koelsch et al. (1991) were modified to measure hexanal concentration by adsorption onto Tenax and desorption using 2-methylbutane. Prior to use, each 10.2 cm metal trap (MSU Biochemistry Shop) was packed with 200 mg of 60/80 mesh Tenax-TA (04916, Alltech Associates, Inc., Deerfield, IL) and each end plugged with silane-treated glass wool (2-0411, Supelco Inc., Bellefonte, PA) (Liu et al., 1992). The packed traps were conditioned with nitrogen gas at a flow rate 25 mL/min at 150°C for at least 12 hr. The conditioned traps were cooled in a dessicator before being attached via a stainless steel Swagelok reducing union to an outlet joint of a purge and trap (P&T) apparatus for volatile collection. To volatilize the flavor compounds, 21 mL of model system homogenate was thawed and combined with 25 mL of PBS in a 500 mL round-bottom flask (605020-1024, Kontes, Vineland, NJ) connected via an L-shaped glass joint to the P&T apparatus. Each solution was sparged with a 30 mL/min nitrogen stream while heating to 60°C on a heating pocket for 90 min. Vercellotti et al. (1992) reported that sparging alone onto Tenax was more efficient at recovering hexanal than purging or sparging under vacuum. To extract the volatile compounds from the Tenax, each trap was pushed into a single-hole rubber stopper and placed in the end of a 15 mL glass graduated centrifuge tube. Using a Pasteur pipet, 2-

methylbutane (27,034-2, HPLC grade, Aldrich Chemical Co., Inc., Milwaukee, WI) was added dropwise to the end of the trap until a total of 1.5-2.0 mL was eluted by centifugation at 80 x g at 4°C. The extracts were transferred into 2 mL concentrator tubes (569812-0002, Kontes), concentrated under nitrogen to 1.0 mL, transferred into 2 mL vials with teflon-lined caps (Supelco, Inc., Bellefonte, PA), and stored in a -20°C freezer. To determine recovery, hexanal was added to 20 mL deionized water and subjected to the isolation and concentration procedure. The recovery was determined to be 81.2% (n = 4; c.v. = 4.77%).

4.3.7 Hexanal Measurement by Gas Chromatography. A Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector was used with a DB-225 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific Inc., Rancho Cordova, CA) to separate hexanal. The column was held at an initial temperature of 35°C for 10 min, then the column temperature was increased at the rates of 3°C per min to 50°C, 6°C per min to 90°C, and 40°C per min to a final temperature of 200°C. The injection port and detector temperatures were 225°C and 275°C, respectively. Helium was used as the carrier gas at a flow rate of 40 mL/min with a 3:1 split ratio. Peak integration was performed by a personal computer running Hewlett-Packard 3365 Series II Chem Station software (HP, Minneapolis, MN). A 10 μL syringe (Hamilton Co., Reno, NV) was used to inject 2 μL aliquots. The syringe and all solutions were held at -20°C to limit volatilization prior to injection. Peak retention times of hexanal reference (H-9008, Sigma) and sample solutions were compared to identify the hexanal elution peaks. The results were calculated as micrograms hexanal per gram of

meat using a standard curve of 3, 6, 20, 60 and 200 μg/mL hexanal in 2-methylbutane. Determinations were made in triplicate.

4.3.8 Statistical Analysis. Statistical analysis was performed using SAS (SAS Institute, Inc., Cary, NC). The general linear model (GLM) procedure was used to perform analysis of variance since the data was unbalanced due to laboratory error. Mean comparisons were performed for the hexanal content data collected. Interactions and contrasts were examined as well. The data collection methods were GC-HS, polyclonal-based ELISA (pELISA) and monoclonal-based ELISA (mELISA). Measurements were taken, in triplicate, every six hours for 36h beginning at time zero. Correlation coefficients were determined between pairs of methods among GC-HS, pELISA, mELISA and TBA-RS considering all seven sampling times.

4.4 RESULTS AND DISCUSSION

4.4.1 Hexanal Measurement by Polyclonal Antibody-based CI-ELISA. Reductive alkylation of CSA and OA with hexanal resulted in 98.1% and 97.7% loss of free amino groups from their native forms, respectively. Degree of modification for the muscle homogenate could not be determined due to incomplete solubility. Maximally-modified CSA, OA and chicken breast homogenate conjugates of 10 mg/mL protein diluted to protein concentrations ranging from 0.003 to 100 μg/mL showed increasing inhibition of binding between antibody and plate-bound antigen, as indicated by decreasing absorbances, with increasing protein concentration (Figure 4.1). An increase in hexanal concentration was concomitant with an increase in protein concentration because the hexanal was covalently bound to the protein. The results take the form of a typical

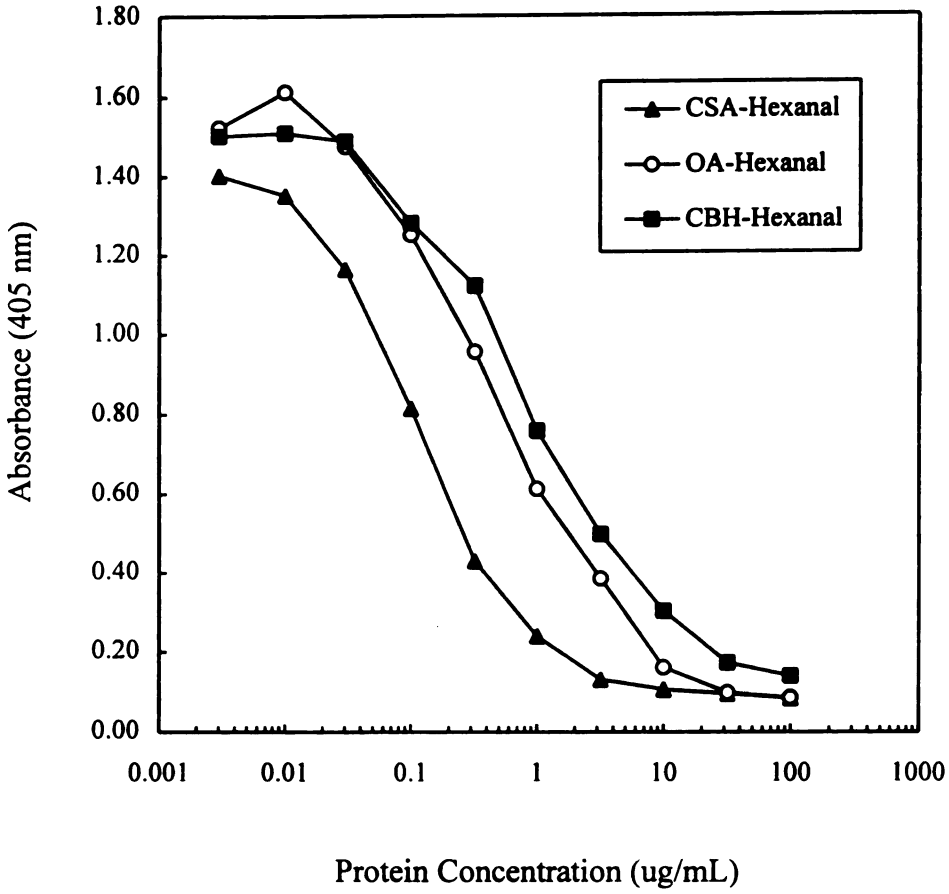


Figure 4.1 Effect of protein (hexanal) concentration on polyclonal antibody binding as measured by CI-ELISA. Solutions of 10 mg/mL protein of chicken serum albumin (CSA), ovalbumin (OA) and chicken breast homogenate (CBH) were maximally-modified with excess hexanal, diluted to protein concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 μg/mL and assayed. Determinations were made in triplicate.

sigmoidal dose-response curve generated over a range of "doses" with fixed antibody concentration (Brady, 1995). The protein concentrations required for 50% inhibition varied among the conjugates, ranging from 0.1 μg/mL for CSA to 1 μg/mL for the chicken breast homogenate. While both OA and CSA showed a 98% loss of reactive amino groups, the absolute number of reactive amino groups likely differs among the proteins. The working range was 0.1-10 μg/mL protein for the chicken breast homogenate versus 0.02-0.5 μg protein per mL for CSA. While the working range of the ELISA was broader for the chicken breast homogenate, the assay was more sensitive for CSA-bound hexanal. The intraplate precision for hexanal-chicken protein adduct added at protein concentrations of 0.1, 1 and 10 g/mL was 4.6, 4.9 and 7.4%, respectively.

Reductive alkylation with different amounts of hexanal at a constant protein concentration of 10 mg/mL yielded proteins with different degrees of amino group modification (Table 4.1). The concentration of reactive amino groups decreased as the amount of added hexanal increased, as measured by TNBS assay. The concentration of reactive amino groups decreased more than 10 times from an initial concentration of 49,000 μM in unmodified OA to 4,090 μM in a 1 μM hexanal-OA solution, resulting in 91.7% modification. As hexanal concentration increased from 1 mM to 25 mM, the degree of modification of amino groups increased only modestly from 91.7 to 98.2%. Over the same range of hexanal concentrations, antibody binding inhibition increased from 11.4% to 91.7%, showing much greater sensitivity to changes in hexanal concentration. This may be due to increasing modification of the immunodominant lysine residues with increasing hexanal addition. Chicken breast homogenate showed the same trend as indicated by decreasing absorbance (Figure 4.2). Therefore, for samples of similar

Table 4.1 Comparison of loss of reactive amino groups and antibody binding inhibition of differentially-modified ovalbumin-hexanal conjugates

Hexanal Concentration[1] (mM)	Reactive Amino Group Concentration[2] (μM)	Amino Group Modification[3] (%)	Binding Inhibition[4] (%)
0	49,000	--	0
1	4,090	91.7	11.4
2	3,720	92.4	17.6
4	3,830	92.2	36.0
6	3,180	93.5	66.0
8	2,900	94.1	79.1
10	2,340	95.2	84.4
15	1,700	96.5	88.2
20	1,030	97.9	90.6
25	901	98.2	91.7

[1] Concentration of hexanal added to solution prior to reduction and dialysis.

[2] Concentration based on leucine standard curve determined by trinitrobenzenesulfonic acid (TNBS) assay.

[3] Defined as loss of reactive amino groups, determined by TNBS assay.

[4] Determined by polyclonal antibody-based competitive indirect-ELISA.

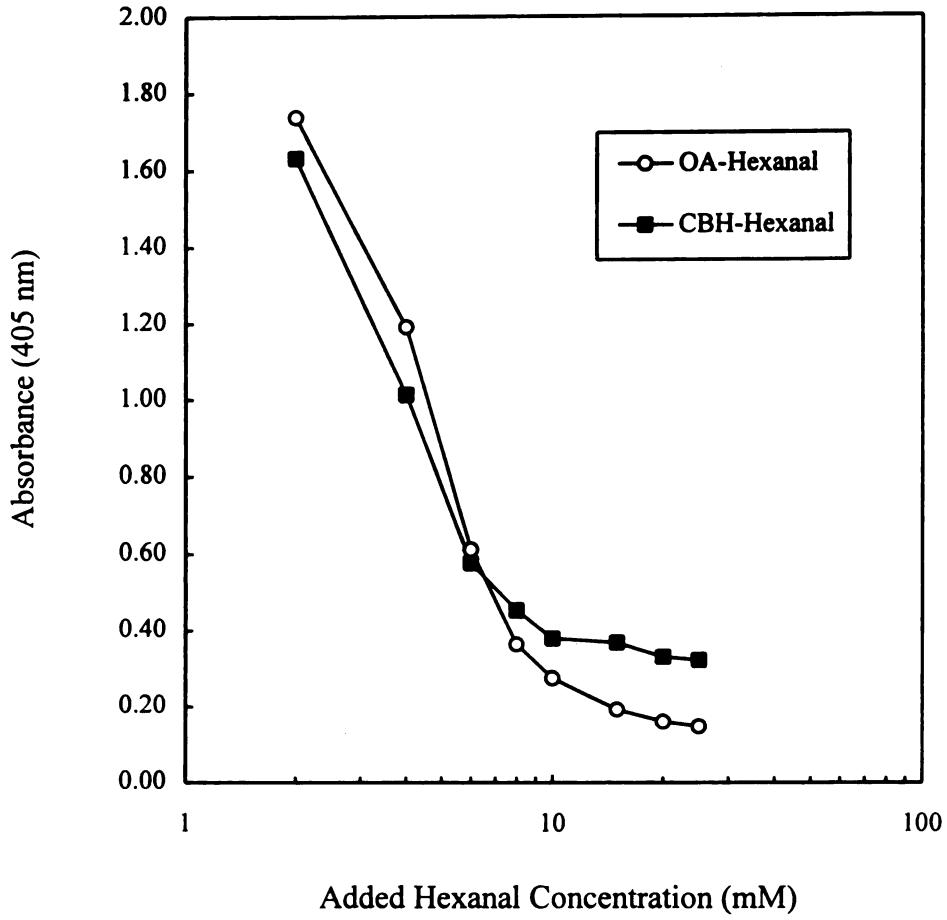


Figure 4.2 Effect of hexanal concentration on polyclonal antibody binding as measured by CI-ELISA. Solutions of 10 mg/mL protein of ovalbumin (OA) and chicken breast homogenate (CBH) were differentially-modified with varying amounts of hexanal. The added hexanal concentrations (prior to reduction, dialysis and dilution) were 2, 4, 6, 8, 10, 15, 20 and 25 mM. The solutions were diluted to 20 μg/mL protein and assayed. Determinations were made in triplicate.

protein concentration modified with different concentrations of hexanal, differences in antibody binding inhibition should be detected.

The relationship between the concentrations of hexanal added and of hexanal bound to the carrier protein and quantified by CI-ELISA was examined. Measured hexanal concentration, based on a standard curve of CSA-hexanal for which the amount of hexanal bound was determined previously by tritium-labeling, was substituted for absorbance (Figure 4.3). Hexanal recoveries, for solutions in the linear portion of the curve, ranged from 3.1-15.4% and increased with increasing hexanal concentration and constant protein concentration. Therefore, not all the added hexanal was detected. This was due, in part, to the loss of some hexanal during dialysis, evident from the odor of the dialysate. However, given that all ovalbumin solutions were extensively modified, the assay may underestimate the amount of hexanal present, at least when hexanal is added at very high (i.e. millimolar) concentrations. Gutheil and Bailey (1992) studied binding of hexanal to muscle myofibrillar proteins in aqueous solutions using an static headspace method and found a similar occurrence. They obtained recoveries of approximately 0.4, 14 and 27% for solutions containing 4.4 mg/mL myosin to which 500, 600 and 700 μg/mL (5, 6 and 7 mM) hexanal had been added. They also considered the effect of differing protein concentrations on hexanal binding and concluded that higher myosin concentrations disproportionately bound more hexanal than lower myosin concentrations, emphasizing the importance of protein concentration when estimating the actual hexanal concentration in a sample.

4.4.2 Comparison of a Monoclonal versus Polyclonal Antibody-based CI-ELISA. Differentially-modified solutions of chicken thigh homogenate were diluted 1:5000, 1:500

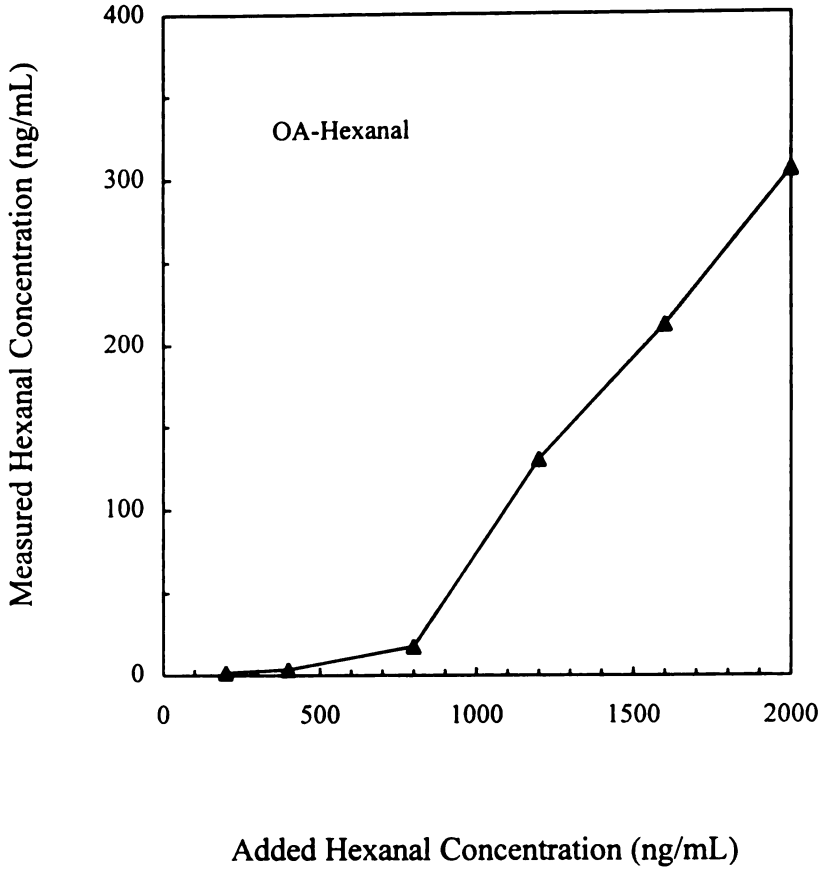


Figure 4.3 Comparison of added and measured hexanal concentrations for hexanal-modified ovalbumin solutions. Solutions of 10 mg/mL protein were combined with varying amounts of hexanal, reduced, dialyzed and diluted 1:500 to a protein concentration of 20 μg/mL. Determinations were made in triplicate by polyclonal antibody-based CI-ELISA.

and 1:50 to achieve solutions of low (2 μg/mL), medium (20 μg/mL) and high (200 μg/mL) protein concentrations. In general, the binding curves of both the polyclonal and monoclonal antibodies were linear from 1 to 10 mMs (100-1000 μg/mL) at low, medium and high protein concentrations (Figure 4.4) . However, the sensitivities of the assays varied. At medium concentration, the range of absorbance units was about 1.3 for both assays. However, at low protein concentration, the polyclonal-based assay spanned 2.0 units, at high concentration, it only covered 0.3 units. The opposite was true of the monoclonal-based assay which spanned 1.2 units at the high protein. This finding suggested that the monoclonal-based assay would provide better sensitivity to detect hexanal in oxidized muscle homogenates, requiring 2000 μg/mL protein to obtain a high enough concentration of hexanal to detect.

4.4.3 Detection of hexanal in oxidized chicken thigh homogenate. Chicken muscle homogenate solutions showed increasing hexanal and malonaldehyde equivalent concentrations, as measured by HS-GC and TBA-RS, respectively, over the first 30 hr followed by a decline (Figure 4.5). The two ELISAs showed increasing hexanal content only to 24 hr. Shahidi and Pegg (1994) observed a linear increase in the hexanal concentration of cooked pork over 6 days of frozen storage followed by a decrease, possibly due to further oxidation of hexanal to hexanoic acid. Both our CI-ELISAs successfully provided quantitative results. The polyclonal-based assay gave values ranging from 0.45 to 2.3 ng/g hexanal; the monoclonal-based assay gave values of 0.97 to 6.4 ng/g. The HS-GC results ranged from 2.0 to 10.4 ng/g. Most of the published works measuring hexanal in meat report GC peak areas or integrator counts limiting comparisons of hexanal contents to within a study. However, several authors reported hexanal

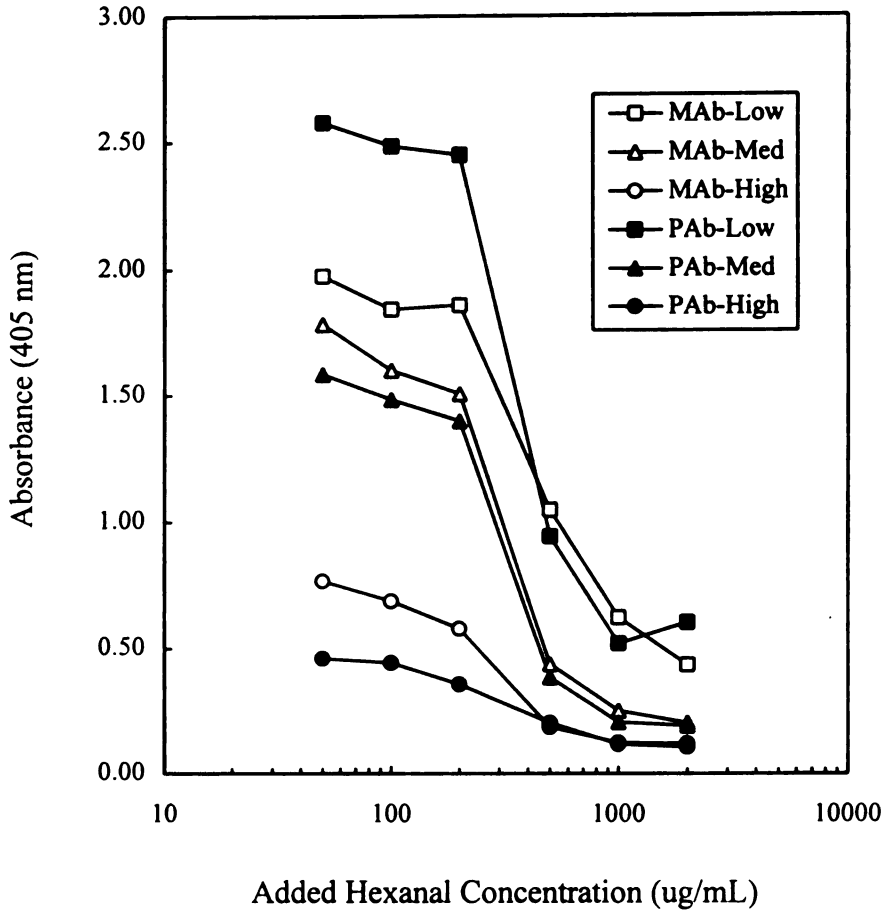


Figure 4.4 Binding curves of differentially-modified chicken thigh homogenate solutions diluted to low (2 μg/mL), medium (20 μg/mL) and high (200 μg/mL) protein concentrations. Solutions had added hexanal concentrations (prior to reduction and dialysis) of 50, 100, 200, 500, 1000, 2000 and 5000 μg/mL. Solutions were subjected to monoclonal antibody (MAb; open symbols)-based and polyclonal antibody (PAb; filled symbols)-based CI-ELISA. Determinations were made in triplicate.

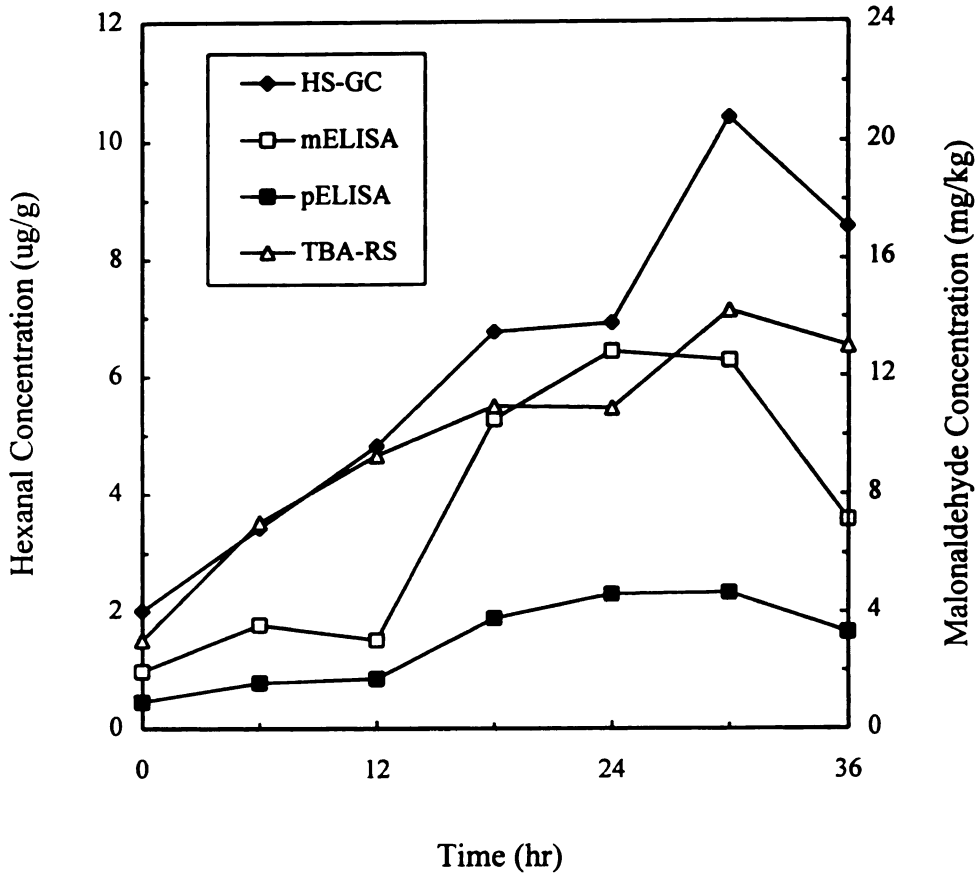


Figure 4.5 Effect of incubation time on hexanal and malonaldehyde concentrations of chicken muscle homogenate as measured by dynamic headspace gas chromatography (HS-GC), monoclonal antibody-based competitive indirect ELISA (mELISA), polyclonal antibody-based competitive indirect ELISA (pELISA) and thiobarbituric acid-reactive substances (TBA-RS) assay. TBA-RS results expressed as milligrams malonaldehyde per kilogram meat. Determinations were made in triplicate.

concentrations based on grams of meat ranging from 30 ng/g to 30 μg/g suggesting our results of 400-1,000 ng/g are in line. Statistically, each method showed a linear rate of change in hexanal content over time. The mELISA also showed a quadratic rate of change in hexanal content over time. The linear rate of change for GC-HS was greater than that for pELISA. Comparisons were made of hexanal contents determined by pELISA and mELISA with GC-HS data at each time point. Hexanal contents determined by mELISA were not significantly different from values determined by HS-GC except at t = 12h ($\alpha = 0.05$). Hexanal contents determined by pELISA were significantly different from values determined by HS-GC at all time points ($\alpha = 0.05$). Therefore, based on the data collected in a meat model system study, the monoclonal-based ELISA may be a suitable alternative to HS-GC analysis for hexanal measurement in a meat system.

Each method showed strong correlation with each of the other methods (Table 4.2). The pair of tests showing the highest correlation was HS-GC and TBA-RS (r = 0.97). Ang and Lyon (1990) reported coefficients of 0.88, 0.90, and 0.92 for cooked and stored broiler thigh, skin, and breast, respectively, comparing values obtained by HS-GC (hexanal content) and TBA methods. Other investigators have also found strong correlations between GC and TBA methods for cooked meats including chicken breast patties (Ang and Young, 1989; Su et al., 1991), ground pork (Shahidi et al., 1987), beef loin slices (Hwang et al., 1990) and roast beef (St. Angelo et al., 1987) with coefficients of 0.86, 0.95, 0.99, 0.81 and 0.92, respectively. Despite the many limitations of TBA assays, they can be used effectively to monitor and evaluate lipid oxidation in meat and other biological tissues when used to assess the extent in general, rather than to quantify

Table 4.2 Correlation coefficients (r) of measurements of the extent of lipid oxidation of chicken thigh homogenate. Methods were headspace gas chromatography (HS-GC), thiobarbituric acid reactive substances (TBA-RS) assay and competitive indirect (CI) ELISA.

	HS-GC	TBA-RS
CI-ELISA (monoclonal)	0.81	0.77
CI-ELISA (polyclonal)	0.89	0.85
HS-GC	--	0.97

malonaldehyde, or when used in combination with a measurement such as hexanal content (Gray et al., 1994).

The dynamic HS-GC method used in this study, while sensitive, was very tedious and time-consuming. The time required for one sample was approximately 2.5 hr, about the same for both the ELISA and the HS-GC methods. However, the GC method was limited by the size of P&T set-up available and by the length of the chromatography run, i.e. the elution time of hexanal, since only one sample can be run at one time. We had a P&T set-up for 3 samples and a run-time of 25 min. Also, we used external standards to calibrate requiring additional time. By ELISA, we were able to run 48 samples including a standard curve, in triplicate, in 2.5 hr, using 2 microtiter plates. Our GC method also required fastidious laboratory practices, including careful cleaning of the glassware and extraction in a cold room to prevent evaporative loss due to the low boiling point of the solvent. Despite the fact that many quality control methods use the dynamic headspace technique (Vercellotti et al., 1992), these methods may not be practical for routine procedures in food-processing plants (Ang et al., 1994). In addition, the occurrence of hexanal-protein binding (Franzen and Kinsella, 1974; Gremli, 1974; Holley et al.,1993; Gutheil and Bailey, 1992; O'Keefe, 1991a,b; Schirle-Keller et al., 1992; Schirle-Keller et al., 1994; Esterbauer et al., 1987) complicates accurate quantitation by HS-GC methods which only measure volatile compounds (Jennings, 1977) neglecting nonvolatiles or volatiles physically bound in the sample (Westendorf, 1985).

The development of objectionable flavors and odors by oxidation has obvious detrimental consequences on food quality and consumer acceptability (Frankel, 1982). Hexanal content has been reported to be a sensitive and reliable indicator for evaluation of

the oxidative state and flavor quality of meat and meat products (Shahidi, 1994). We have shown that use of a hexanal-specific antibody-based ELISA to monitor lipid oxidation in chicken thigh homogenate is a faster and simpler alternative to dynamic HS-GC.

CHAPTER 5

CONCLUSIONS

1. We successfully produced highly sensitive and specific polyclonal antibodies which react primarily with hexanal-lysine adducts. The immunogen was bovine serum albumin modified extensively with hexanal by reductive alkylation using sodium cyanoborohydride as the reducing agent. By selecting a reducing agent specific for Schiff bases, we were able to limit the products of the protein-hexanal reaction to lysine derivatives. By using an excess of hexanal, we were able to promote predominantly hexanal-modified lysine residues as the antigenic determinant.

2. We found only two compounds to cause cross-reactivity with our polyclonal antiserum against hexanal-modified bovine serum albumin at a level above 2%. The compounds were heptanal and pentanal, the aliphatic aldehydes with one greater and one fewer carbons than hexanal, respectively. Because both compounds are also lipid oxidation breakdown products, our assay remains specific for monitoring lipid oxidation. In addition, neither heptanal nor pentanal is generally found in foods at concentrations as high as the concentrations at which hexanal may occur.

3. We established that our polyclonal antibody-based immunoassay can measure hexanal concentrations more rapidly and simply than a dynamic headspace gas chromatography method. In addition, the ELISA correlated well with both GC and TBA methods. While not as sensitive as the monoclonal antibody-based assay, the polyclonal-based assay was able to detect differences in the hexanal concentrations of chicken thigh muscle homogenate incubated over 36 hr and is a viable alternative method to dynamic headspace gas chromatography.

CHAPTER 6

FUTURE RESEARCH

The findings of the preceding study suggest that further research is warranted. The sensitivity of the polyclonal antiserum may be increased by performing immunoaffinity purification on an antigen column to select for the antibodies with more affinity for a particular antigen, such as oxidized chicken protein. Another approach to improve assay sensitivity is to use a direct competitive format instead of the indirect format. This requires either: 1) conjugation of hexanal to horseradish peroxidase (HRP) to compete with antigen-containing extract for plate-bound antibody or 2) conjugation of polyclonal antibody and HRP for which antigen-containing extract and plate-bound antigen would compete. Also, further optimization of the assay may be attempted, particularly the use of another substrate, such as 3,3',5,5'-tetramethylbenzidine (TMB; Deshpande, 1996) or o-phenylene diamine (OPD; Kemeny, 1991), or another enzyme-substrate pair.

Additional experimentation is necessary to develop a clearer understanding of hexanal-muscle protein binding. Once a model is established, factors corresponding to protein concentration can be determined to adjust for hexanal present but not detected.

To continue pursuing application of our ELISA within the meat industry, the next step would be to move from a model system to an actual muscle or meat product. Appropriate extraction procedures must be developed for different products, such as raw or cooked meats, and whole muscle, formed or comminuted products. Use of the assay

should not be limited to the product itself. Raw materials to be used in formed and comminuted products could also be evaluated for lipid oxidation. Extenders, such as soy protein, are known to contain hexanal imparting off-flavor (MacLeod and Ames, 1988). Other sources of off-flavor may be in the production process. Chiller water of commercial poultry processors, used to decrease carcass temperature, has been found to contain aldehydes, including hexanal (Tsai et al., 1986). However, the latter application would require a "carrier" for the hexanal since the antibodies do not recognize free hexanal.

Results of a preliminary study suggest that tris(hydroxymethyl)aminomethane (Tris) buffer used in place of protein may function as the necessary carrier for hexanal. Use of Tris buffer could be specified in the protocol as a sample preparation reagent for low protein or nonprotein samples. This approach may permit application of our ELISA to vegetable oils (Reineccius, 1996) and potato products (Salinas et al., 1994) for which hexanal has long been an indicator of lipid oxidation.

While several published studies have reported strong correlations between the development of hexanal in meat and sensory data, the researchers measured volatile hexanal using gas chromatography. In addition to the fact that the use of an ELISA to measure hexanal is a novel approach, both the polyclonal-based assay and the monoclonal-based assay measure total hexanal (volatile and nonvolatile). Therefore, sensory evaluation should be included in future studies of meat or other foods.

A broader reaching, but nonetheless reasonable application of our assay within the poultry industry is for assessment of turkey spermatozoa quality. Most turkeys are bred by artificial insemination because the size of the turkey male attained through genetic selection makes them unable to perform natural mating (Sexton, 1979). Formation of lipid

peroxides limits survival of spermatozoa during in vitro storage (Wishart, 1989) and TBA-RS assays are used to monitor extent of lipid peroxidation (Cecil and Bakst, 1993). Spermatozoa, particularly fowl and turkey, contain high concentrations of arachidonic acid, a hexanal precursor (Darin-Bennett et al., 1974). Hexanal measurement as an alternative to TBA-RS assay to assess extent of lipid oxidation in biological samples has already been successful (Frankel et al., 1992). In addition, Jones et al. (1979) stated that measurement of the extent of lipid peroxidation may provide a useful basis for biochemical appraisal of human sperm quality.

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