Production and Specificity of Polyclonal Antibodies to Hexanal−Lysine Adducts

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Hexanal content is a widely used index of lipid oxidation in foods. The objectives of this study were to develop antibodies to hexanal−lysine adducts, devise an ELISA, and characterize antibody specificity. Hexanal was made immunogenic by covalent attachment to lysine side chains of bovine serum albumin via reductive alkylation. Polyclonal antibodies had antiserum titers as high as 6.15×10^5. A competitive indirect ELISA was developed with a detection limit of 0.7 ng of hexanal/mL. Antibodies were carrier-independent, reacting with hexanal conjugates of several proteins but not with the corresponding native proteins. Cross-reactivities with chicken serum albumin conjugates of *n*-heptanal, *n*-pentanal, and *n*-octanal were 86.3, 11.8, and 2.2%, respectively. Antibodies reacted strongly with hexanal-modified lysine and hexanal-modified ϵ-aminocaproic acid but did not recognize free amino acids or free hexanal. It may be feasible to use this ELISA to monitor lipid oxidation in food provided hexanal is alkylated to a carrier protein prior to analysis.

Keywords: *Immunoassay; hexanal; polyclonal antibodies; lipid oxidation*

INTRODUCTION

Lipid oxidation leads to the destruction of lipids in biological and food systems (Kappus, 1991). A number of diseases have been related to lipid peroxidation including cancer (Kappus, 1991), rheumatoid arthritis (Lopez-Bote et al., 1993), Parkinson's disease (Yoritaka et al., 1996), and atherogenesis (Palinski et al., 1995). Rancidity arising from lipid oxidation is often the decisive factor in determining food product storage life (Frankel, 1993). Unfortunately, the most widely used assay for measuring lipid peroxidation, the 2-thiobarbituric acid test, is not specific for oxidation products because many other compounds are reactive with 2-thiobarbituric acid (Gutteridge and Halliwell, 1990) and it is not suitable for oxidation products of the common fatty acids, oleic and linoleic (Frankel, 1993).

Hexanal content is a popular index of lipid oxidation in foods. Hexanal is a 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 of oxidized or rancid foods (Gray and Monahan, 1992). Hexanal measurement by headspace gas chromatography has been used successfully to monitor lipid oxidation in biological specimens, such as liver homogenate (Frankel et al., 1989) and plasma lipoproteins (Walzem et al., 1995), as well as in a variety of foods (Shahidi, 1994). Currently, neither a standard method for the quantitation of hexanal nor a convention for the reporting of hexanal data exists, making comparison of results difficult. Covalent binding between hexanal and proteins in food and other biological systems may prevent volatilization of hexanal, thus further complicating accurate quantification by headspace gas chromatographic methods (Franzen and Kinsella, 1974; Gremli, 1974; O'Keefe, 1991).

Immunoassays are becoming increasingly common, offering both analytical and cost-savings benefits over conventional techniques, including sensitivity, specificity, speed, and simultaneous testing of numerous samples (Samarajeewa et al., 1991; Deshpande, 1996). Protein modifications as simple as ethylation and methylation have resulted in production of specific antibodies against acetaldehyde-modified and formaldehyde-modified protein, respectively, and not against epitopes of the unmodified protein (Steinbrecher et al., 1984). Antibodies have also been developed to putative aldehydic products of lipid oxidation including malonaldehyde and 4-hydroxynonenal (Palinski et al., 1989; Petit et al., 1995).

The primary goal of this study was to produce and characterize polyclonal antibodies specific to hexanal−lysine adducts to be incorporated into an enzyme-linked immunosorbent assay (ELISA). The specific objectives were (1) to covalently bind hexanal to a carrier protein, (2) to produce polyclonal antibodies and develop an ELISA, and (3) to characterize the antibodies for protein, aldehyde, and amino acid specificities by assessing cross-reactivity with compounds structurally similar to the antigen. It is hoped that this work will ultimately lead to the development of an ELISA to quantify hexanal as an index of lipid oxidation in foods and other biological systems.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), chicken serum albumin (CSA), high molecular weight protein standards, sodium cyanoborohydride ($NaCNBH_3$), propanal, pentanal, hexanal, octanal, trinitrobenzenesulfonic acid (TNBS), ϵ-aminocaproic acid, γ-aminobutanoic acid, Freund's complete adjuvant, Freund's incomplete adjuvant, polyoxyethylenesorbitan monolaurate (Tween 20),

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2,2′-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), and caproic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Butanal, heptanal, nonanal, *trans*-2-hexenal, 2-methylpentanal, 2-methylbutanal, 3-methylbutanal, 2-heptanone, 2-hexanol, and *n*-hexanol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Goat anti-rabbit IgG−horseradish peroxidase (IgG−HRP) conjugate was purchased from Organon Teknika Corp. (West Chester, PA). Immunlon 2 Removawell strips and Removawell strip holders were purchased from Dynatech Laboratories, Inc. (Chantilly, VA). All other chemicals were of reagent grade or better.

Protein−Hapten Conjugation by Reductive Alkylation. Alkylation of proteins and amino acids was based on the method of Steinbrecher et al. (1984) with the following modifications: (1) reactant concentrations were 200 mM aldehyde, 15 mg/mL protein, and 80 mM $NaCNBH_3$; (2) aldehyde was added, as one aliquot, immediately prior to $NaCNBH_3$; and (3) reaction was run at ambient temperature and a basic pH to accelerate the reaction (Jentoft and Dearborn, 1979). In a typical reaction, 246 μL of hexanal was added to 9 mL of 16.7 mg/mL BSA diluted in 0.1 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4 (PBS). Reducing conditions at pH 8.5−9.0 were established by adding 1 mL of 800 mM $NaCNBH_3$ in 0.1 N NaOH to the hexanal−BSA solution. Excess reagents were removed by dialysis against two changes of PBS at ambient temperature. The same protocol was used to modify KLH, CSA, and the high molecular weight protein standards. The reductive alkylation protocol was also used to modify CSA with other aldehydes, ketones, and alcohols: propanal, butanal, pentanal, heptanal, octanal, nonanal, *trans*-2-hexenal, 2-methylpentanal, 2-methylbutanal, 3-methylbutanal, 2-heptanone, 2-hexanol, and *n*-hexanol.

The protocol was revised to modify amino acids with hexanal. The reactant concentrations were 200 mM hexanal, 20 mM $NaCNBH_3$, and 200 mM amino acid, and dialysis was not performed due to the small size of the molecules. The modified amino acids were L-lysine monohydrochloride, D-lysine monohydrochloride, L-arginine, glycine, and ϵ-aminocaproic acid. Octanal was substituted for hexanal for reductive alkylation with γ-aminobutanoic acid.

Lysine modification achieved in each reaction was determined by measuring the loss of reactive amino groups in a protein by the TNBS acid assay (Habeeb, 1966) using a standard curve of leucine solutions ranging in concentration from 10 to 300 μM to determine moles of reactive amino groups per mole of protein (Adler-Nissen, 1979). Modification was expressed as the percentage decrease in TNBS-reactive amino groups of native protein.

Polyclonal Antibody Production. Three female rabbits (white New Zealand, 3 months old) from Hazelton Laboratories (Kalamazoo, MI) were housed by the Michigan State University Laboratory Animal Resources and handled according to the Rabbit Antibody Production Service program. Rabbits, identified as 45903, 45904, 45911, were immunized subcutaneously with a total of 1 mL of antigen−adjuvant emulsion administered in 8−10 sites on their backs. The initial injection consisted of 500 μg of hexanal-modified BSA in 0.5 mL of 0.85% sterile saline microemulsified with 0.5 mL of Freund's complete adjuvant per rabbit. Booster injections were administered at 4, 8, 18, and 75 weeks and consisted of 500 μg of hexanal-modified BSA emulsified with Freund's incomplete adjuvant. 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, pH 7.6. Briefly, the blood was allowed to coagulate at ambient temperature for 2 h and the clot to contract at 4 °C overnight. Next, the blood was decanted into a centrifuge tube and spun at 10000*g* for 20 min at 4 °C. Saturated ammonium sulfate was added dropwise to the supernatant to achieve 30% saturation. The solution was centrifuged at 10000*g* for 20 min at 4 °C, the pellet dissolved to the original volume in PBS, diluted 2:1 with saturated ammonium sulfate, 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 dialysis at 4 °C against 4 L of PBS for 3 days, with buffer changes every 24 h. Sera were diluted to the original volume with PBS and frozen for storage.

Indirect ELISA for Serum Titer Determination. Serum titers for each bleeding were determined by indirect ELISA. Immunlon 2 microtiter plates were coated with 100 μL per well of 2 μg/mL hexanal-modified CSA in 0.1 M carbonate buffer, pH 9.6, held at 4 °C for 16 h, and washed four times with 0.05% Tween 20 in PBS (PBS−Tween) using an automatic plate washer (model ELP-40, Bio-Tek Instruments, Inc., Winooski, VT). Each plate was blocked with 300 μL per well of 0.5% casein in PBS (PBS−casein), incubated at 37 °C for 30 min, and washed four times. Sera were diluted in PBS−casein from 10^{-3} to 10^{-6}, and 50 μL per well of each solution was applied to a plate in triplicate wells. Each plate was incubated for 60 min at 37 °C and washed four times. Bound polyclonal antibodies were labeled by adding 100 μL per well of goat anti-rabbit IgG−HRP diluted 1:500 in casein−PBS, incubated for 30 min at 37 °C, and washed eight times. A substrate solution consisting of 1 mL of 300 μg/mL ABTS, 11 mL of citrate buffer, pH 4.0, and 8 μL of concentrated hydrogen peroxide was prepared and 100 μL added 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 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.

Antibody Specificity by ELISA. A competitive indirect (CI) ELISA was developed to determine the protein, aldehyde, and amino acid specificities of the antibodies by comparing the reactivities of both free and modified amino acids, proteins, and aldehydes. 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 protocol was the same as that of 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 free hexanal, 0, 10, 20, and 30% methanol in PBS was used for sample dilutions of hexanal and hexanal−CSA conjugate. Conjugate concentrations are given as protein concentration (micrograms per milliliter) in experiments with native and modified proteins. Conjugate concentrations are given as amino acid concentration (millimoles per liter) in amino acid specificity experiments. The hexanal concentration of CSA−hexanal conjugate was calculated on the basis of the percentage lysine modification determined using the TNBS assay. Therefore, when antiserum reactivity was tested with unmodified and hexanal-modified CSA, hexanal concentration (nanograms per milliliter) was used. All experiments were run in triplicate. Standard error of the mean was determined for each analyte using one-way analysis of variance (JMP software, version 3, SAS Institute, Inc., Cary, NC).

Assay Sensitivity and Precision. To establish the working range of the CI-ELISA, a standard curve of hexanal−CSA adduct was produced by diluting a stock solution of 15 mg/mL protein to concentrations ranging from 0.01 to 32 μg/mL. Intraplate and interplate assay precisions of the 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 (Deshpande, 1996).

Western Blot. The antibody specificities to unmodified and hexanal-modified proteins were evaluated on the basis of the Western blot method of Wang et al. (1992). Unmodified and hexanal-modified BSA, CSA, and high molecular weight standard proteins were diluted in PBS to protein concentrations of 100, 100, and 250 μg/mL, respectively. The proteins were transferred electrophoretically from a 12% acrylamide

Table 1. Serum Titers of Polyclonal Antibodies against Hexanal-Modified BSA in Rabbits[a]

	titer × 10^5		
week[b]	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	

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

SDS−PAGE gel to a nitrocellulose membrane (BA-S 85, Schleicher and Schuell, Keene, NH) using a Mini-Protein gel assembly (Bio-Rad Laboratories, Hercules, CA) for 1 h at 350 mA and 100 V. The membrane was washed with PBS−Tween, blocked for 30 min with casein−PBS, and washed again. The membrane was incubated at ambient temperature with antiserum diluted in PBS−casein 1:2000 for 30 min and washed. The membrane was then incubated with IgG−HRP conjugate diluted in PBS−casein 1:2000 for 10 min and washed. Finally, the membrane was incubated with substrate solution and stopped after 7 min with deionized water.

RESULTS AND DISCUSSION

Preparation of Hexanal Conjugates for Antibody Production. The percentage lysine modification of the different hexanal−BSA conjugate preparations used in antibody production ranged from 97.1 to 98.4% for the primary and booster injections. Zielinski (1998) found strong agreement between percentage lysine modification determined using amino acid analysis and the TNBS assay for hexanal conjugates of CSA, BSA, and KLH. In that study, only lysine residues of protein were modified by reductive alkylation. Similarly, Weisgraber et al. (1978) subjected low-density lipoprotein to reductive methylation using formaldehyde and compared the amino acid compositions of the native and modified solutions. They reported modification of 18 of 20 lysine residues and no changes in any other residues. 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. These findings were expected because the reducing agent, $NaCNBH_3$, 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).

Antiserum Titers. Polyclonal antisera that 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, respectively, following the second boost (Table 1). Rabbit 45911 produced its highest serum titer, 1.64×10^5, following the third boost. Palinski et al. (1989) reported titers of $>10^5$ for polyclonal antisera to malonaldehyde-modified low-density lipoprotein after a primary and two booster injections. The antiserum collected following the second boost of rabbit 45903 was used for all subsequent testing because it had one of the highest titers.

Assay Sensitivity and Precision. A standard curve of hexanal concentration versus absorbance was generated by substituting hexanal concentration for protein concentration based on the amount of hexanal bound (95.3% modification) to CSA during reductive alkylation as determined by TNBS assay (Figure 1). The limit of detection, defined as 2 times the standard deviation of the mean response of the zero standard (Deshpande, 1996), was determined to be 0.7 ng of hexanal/mL using hexanal-modified CSA in the CI-ELISA. The intraplate coefficient of variation values for hexanal−CSA 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 coefficients of variation for hexanal−CSA 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. These results suggest that the ELISA can detect hexanal at concentrations similar to or lower than conventional headspace gas chromatographic methods (Vercellotti, 1992; Reineccius, 1996).

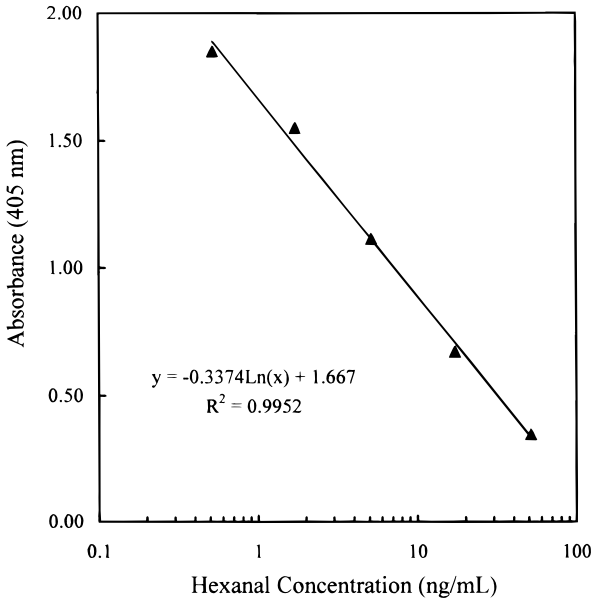

Figure 1. Standard curve of hexanal-modified CSA using polyclonal antibodies against hexanal-modified BSA in a CI-ELISA. The equivalent hexanal concentration of the CSA conjugate was calculated from the protein concentration and percentage modification of CSA lysine residues by hexanal. Standard error of the means was 0.02 absorbance unit.

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 used in the CI-ELISA were 95.3, 86.8, and 94.8%, respectively. 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 in the CI-ELISA (Figure 2). Lack of recognition of unmodified BSA, the immunization carrier protein, suggests that the protein was sufficiently modified to present primarily hexanal moieties as the antigenic determinants. Hexanal-modified KLH required a concentration of ∼30 times that of modified CSA or modified BSA to achieve 50% binding inhibition, likely due to the lower degree of amino group modification and fewer available lysine residues per mole of KLH (Zielinski, 1998).

A Western blot of unmodified and hexanal-modified BSA, CSA, and the high molecular weight protein mixture showed antiserum recognition of the hexanal-modified proteins but not the unmodified forms (Figure 3), confirming the results of the ELISA. Hexanal-

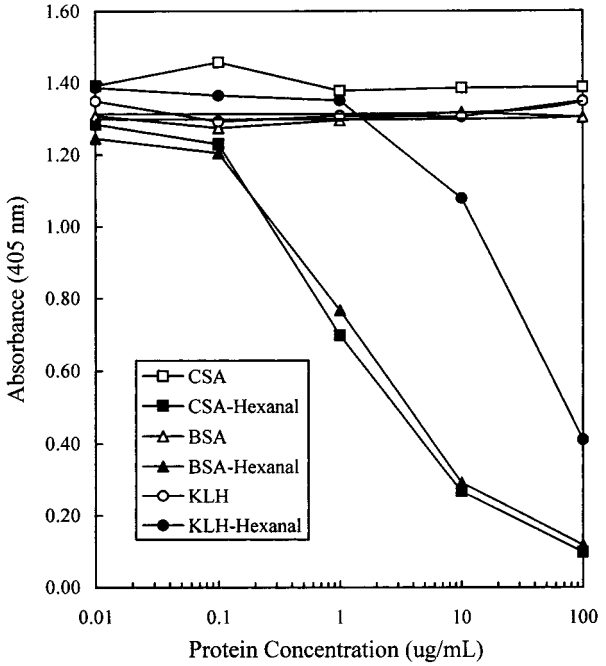

Figure 2. Protein specificity of antibodies to hexanal-modified BSA determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified CSA−hexanal and incubated with antiserum diluted 1:3160. Unmodified and modified BSA, CSA, and KLH competed at concentrations of 0.01, 0.1, 1, 10, and 100 μg/mL. Standard errors of the means ($n = 3$) were 0.02, 0.03, 0.01, 0.03, 0.01, and 0.05 absorbance unit for CSA, CSA−hexanal, BSA, BSA−hexanal, KLH, and KLH−hexanal, respectively.

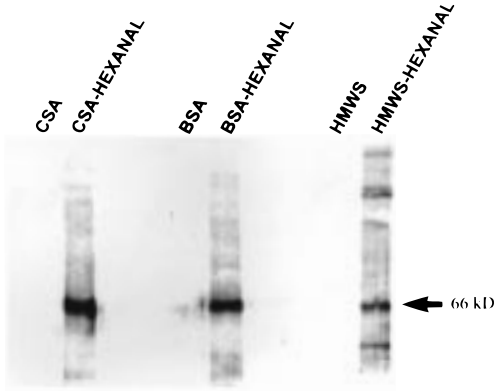

Figure 3. Western blot of unmodified and hexanal-modified CSA (lanes 1 and 2), BSA (lanes 3 and 4), and high molecular weight standard (HMWS; lanes 5 and 6) containing myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), and ovalbumin (45 kDa).

modified proteins reacted with antiserum regardless of size. When a mixture of proteins was modified with hexanal, bands were visible for myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), and ovalbumin (45 kDa), suggesting the immunodominant 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 4). Hexanal−protein conjugates in 10, 20, and 30% methanol showed little difference in binding from the control (no methanol), confirming that methanol did not interfere with the assay. This finding suggests the antiserum recognizes an epitope larger that the six-carbon hexanal molecule.

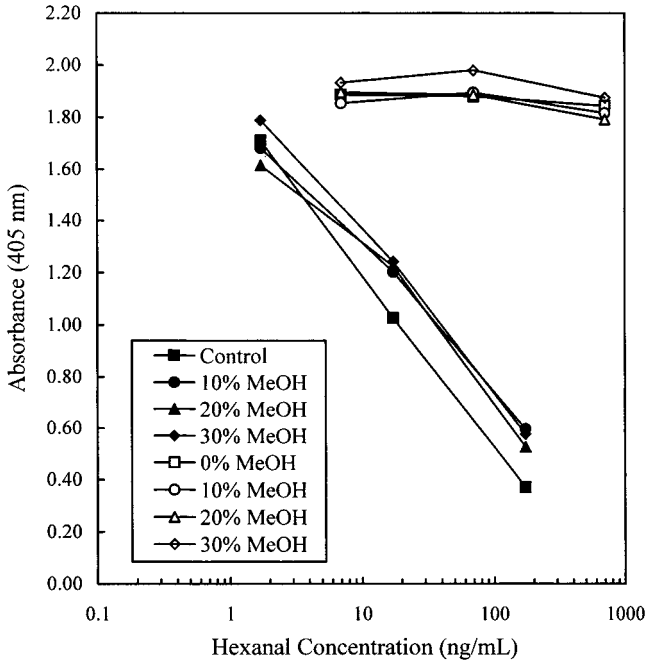

Figure 4. Specificity of antibodies to hexanal-modified BSA determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified CSA and incubated with antiserum diluted 1:3160. Free hexanal (open symbols) or CSA-conjugated hexanal (solid symbols) competed in 0, 10, 20, or 30% methanol. Standard errors of the means ($n = 3$) were 0.02, 0.02, 0.02, and 0.04 absorbance unit for CSA−hexanal conjugates in 0, 10, 20, and 30% methanol, respectively. Standard errors of the means ($n = 3$) were 0.02, 0.04 0.02, and 0.02 for hexanal in 0, 10, 20, and 30% methanol, respectively.

Table 2. Percentage Modification of Reactive Amino Groups of CSA by Aldehydes during Reductive Alkylation

compound	modification[a] (%)	compound	modification[a] (%)
propanal−CSA	92.9	nonanal−CSA	90.8
butanal−CSA	92.5	2-*trans*-hexenal−CSA	98.1
pentanal−CSA	93.1	2-methylpentanal	97.0
hexanal−CSA	92.6	2-methylbutanal	97.0
heptanal−CSA	93.4	3-methylpentanal	96.2
octanal−CSA	92.7		

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

Aldehyde Specificity. CSA was modified by reductive alkylation with various aldehydes. Modification of the lysine residues of CSA ranged from 90.8 to 98.1% depending on the aldehyde used (Table 2). The alkylation procedure was not optimized for each aldehyde and may be one explanation as to why percentage modification was variable.

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 5). Heptanal-modified CSA showed the highest cross-reactivity at 86.3%, followed by pentanal- and octanal-modified CSA with cross-reactivities of 11.8 and 1.9%, respectively (Table 3). The differences in antiserum reactivity suggest that antibodies can discriminate between differences in the carbon number of the aldehyde. Similar percentage modification of CSA by each aliphatic aldehyde suggests that differences in binding inhibition are due to epitope structure and not to differences in epitope density.

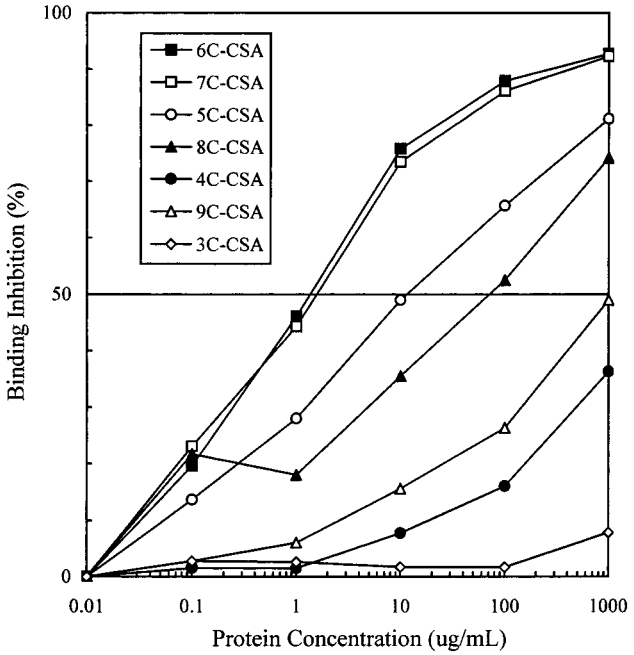

Figure 5. Aliphatic aldehyde specificity of antibodies to hexanal-modified BSA determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified CSA and incubated with antiserum diluted 1:3160. Aldehyde-modified CSA competed at protein concentrations of 0.01, 0.1, 1, 10, 100, and 1000 μg/mL. Standard errors of the means ($n = 3$) were 2.2, 3.1, 2.6, 2.0, 2.9, 2.8, and 2.8% for propanal (3C), butanal (4C), pentanal (5C), hexanal (6C), heptanal (7C), octanal (8C), and nonanal (9C), respectively.

Table 3. Cross-Reactivity of Polyclonal Antibodies against Hexanal-Modified BSA with Aldehyde-Modified CSA by CI-ELISA

aldehyde component	chemical structure	cross-reactivity[a] (%)
hexanal	CSA−NH$(CH_2)_5CH_3$	100.0
2-*trans*-hexenal	CSA−NHCHCH=CH$(CH_2)_2CH_3$	100.0
heptanal	CSA−NH$(CH_2)_6CH_3$	86.3
pentanal	CSA−NH$(CH_2)_4CH_3$	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−NH$CH_2CH(CH_3)(CH_2)_2CH_3$	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−NH$CH_2CH(CH_3)CH_2CH_3$	<0.1

[a] Defined as concentration of hexanal−CSA required for 50% inhibition divided by concentration of aldehyde−CSA required for 50% inhibition multiplied by 100.

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, propanal, and butanal adducts, respectively. Perata et al. (1992) reported that antiserum to acetaldehyde-modified KLH exhibited cross-reactivities of 6.6, 100, and 106% when BSA was modified with formaldehyde, acetaldehyde, and propanal, respectively. The authors observed no cross-reactivity with butanal or pentanal. In another study, an antiserum to carbamylated low-density lipoproteins was produced (Steinbrecher et al., 1984), which recognized homocitrulline (ϵ-carbamyldiaminocaproic acid), a lysine derivative. Homocitrulline was highly competitive with carbamylated low-density lipoproteins,

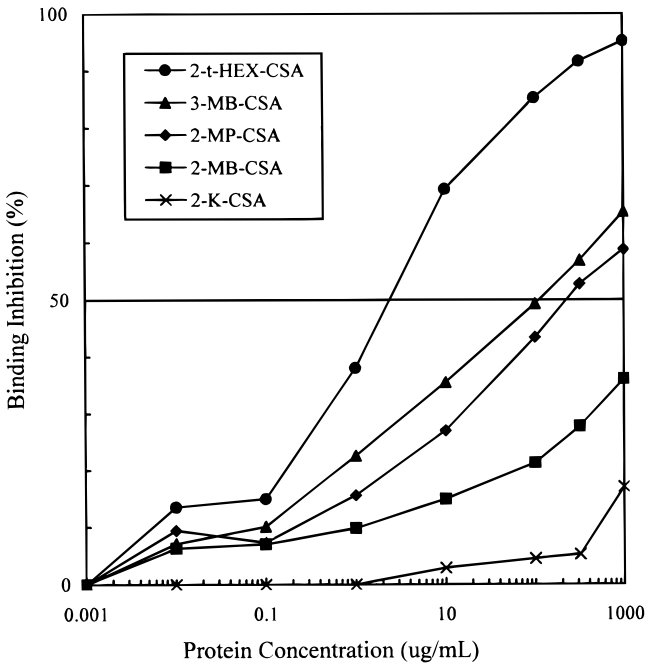

Figure 6. Carbonyl compound specificity of antibodies to hexanal-modified BSA determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified CSA and incubated with antiserum diluted 1:3160. Modified CSA competed at protein concentrations of 0.001, 0.01, 0.1, 1, 10, 100, and 1000 μg/mL. Standard errors of the means ($n = 3$) were 1.4, 2.1, 1.6, 2.0, and 1.1% for 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), respectively.

whereas citrulline (δ-carbamyldiaminopentanoic acid), containing one fewer carbon, was a very weak competitor.

The competitive abilities of other carbonyl compounds were considered as well. CSA conjugates of the Strecker aldehydes, 2-methylbutanal, 2-methylpentanal, and 3-methylbutanal, were evaluated for cross-reactivity. These branched aldehyde conjugates (all >96.2% modification) were considerably less effective competitors than the aliphatic ones (Figure 6). The cross-reactivities of 3-methylbutanal- and 2-methylpentanal-modified CSA were 2.2 and 1.1%, respectively (Table 3). In addition, conjugates of 2-heptanone, *n*-hexanol, 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% when conjugated to CSA (98.1% modification), likely due to its length and to reduction of its double bond during reductive alkylation.

Amino Acid Specificity. Hexanal conjugates of the amino acids glycine, lysine (α,ϵ-diaminocaproic acid), and arginine and the lysine derivativee ϵ-aminocaproic acid (does not contain a free α-amino group) were evaluated for competitiveness by ELISA. Although neither the free amino acids nor free ϵ-aminocaproic acid was recognized by the antiserum, hexanal-modified ϵ-aminocaproic acid was a highly effective competitor and, in fact, a better competitor than hexanal-modified lysine (Figure 7). The concentration required for 50% antibody binding inhibition was 1091 mM for lysine−

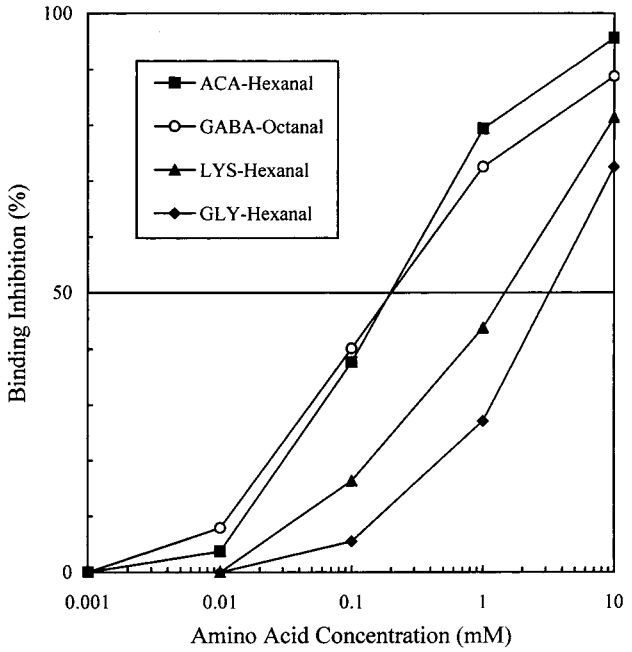

Figure 7. Acid specificity of antibodies to hexanal-modified BSA determined by CI-ELISA. Microtiter wells were coated with 2 μg/mL hexanal-modified CSA and incubated with antiserum diluted 1:3160. Conjugates competed at concentrations of 0.001, 0.01, 0.1, 1, and 10 mM. Standard errors of the means ($n = 3$) were 0.85, 1.2, 1.5, and 1.4% for ϵ-aminocaproic acid (ACA)−hexanal, γ-aminobutanoic acid (GABA)−octanal, lysine (LYS)−hexanal, and glycine (GLY)−hexanal conjugates, respectively.

Table 4. Reactivity of Polyclonal Antibodies against Hexanal-Modified BSA with Aldehyde−Amino Acid Conjugates by CI-ELISA

conjugate	concn required for 50% inhibition (mM)
ϵ-aminocaproic acid−hexanal	234
γ-aminobutanoic acid−octanal	260
lysine−hexanal	1090
glycine−hexanal	3090

hexanal conjugate compared to 234 mM for ϵ-aminocaproic acid conjugate (Table 4). The lower reactivity of modified lysine may be due to steric hindrance caused by the proximity of the α- and ϵ-amino groups. Palinski et al. (1989) reported that malonaldehyde-modified ϵ-aminocaproic acid and malonaldehyde-modified *tert*-butoxycarbonyllysine could both bind antibodies to malonaldehyde-modified low-density lipoproteins.

Hexanal-modified glycine and arginine were considerably less competitive. Their reactivity is likely attributable to modification of the α-amino groups that 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 that of N^{α}-acetyllysine.

Although the length of the epitope (number of atoms) influences the ability of the antibodies to recognize the structure, the presence of a nitrogen atom may also be important. γ-Aminobutanoic acid was modified with octanal to achieve a molecule of length equal to that of hexanal-modified ϵ-aminocaproic acid. The difference in structures between hexanal−ϵ-aminocaproic acid [CH_3-$(CH_2)_5NH(CH_2)_5COOH$] and octanal−γ-aminobutanoic [$CH_3(CH_2)_7NH(CH_2)_3COOH$] was in the position of the nitrogen atom. Both molecules competed equally well in the ELISA (Figure 7). However, the 13-carbon carboxylic acid, tridecanoic acid, was not reactive, suggesting the requirement of a nitrogen atom for reactivity with these antibodies. We propose that the immunodominant epitope is a hexanal-modified lysine residue within the protein chain.

These experimental findings indicate that the antiserum recognizes protein-bound hexanal regardless of the protein carrier and can discriminate differences as small as one carbon in the aldehyde chain length. Results further 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. Prior to use as an analytical assay, procedures will need to be devised that will react hexanal in the sample to endogenous or added protein during extraction. Although the antibodies were not specific to hexanal, cross-reactivity with heptanal and pentanal should not compromise the usefulness of the ELISA for monitoring lipid oxidation. All three aldehydes are lipid oxidation products, so cross-reactivity with these aldehydes will not make the assay nonspecific for lipid oxidation. Development of an ELISA incorporating these antibodies to monitor lipid oxidation in food by measuring the hexanal content, therefore, seems feasible.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis[3-ethylbenzthiazoline-6-sulfonic acid]; BSA, bovine serum albumin; CSA, chicken serum albumin; CI-ELISA, competitive indirect enzyme-linked immunosorbent assay; IgG−HRP, immunoglobulin G−horseradish peroxidase conjugate; KLH, keyhole limpet hemocyanin; PBS, phosphate buffer; TNBS, trinitrobenzenesulfonic acid.

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