JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Reaction Products Formed in a Model Reaction between Pentanal and Lysine-Containing Oligopeptides

TRINE K. DALSGAARD, JACOB H. NIELSEN, AND LOTTE B. LARSEN*

Danish Institute of Agricultural Sciences, Department of Food Science, Research Centre Foulum, P.O. Box 50, DK-8830 Tjele, Denmark

Aldehydes formed as a result of lipid oxidation form fluorophores after binding to proteins. The structure of the fluorophores formed by reaction between saturated aldehydes and lysine has not yet been identified. The reaction products formed in the reaction between pentanal and oligopeptides were studied by fluorescence spectroscopy and mass spectrometry. The emission spectra showed an increase in fluorescence intensity with incubation time, and the rates were linear with the concentration of pentanal. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analyses of the reaction products suggested a molar relation for peptide:pentanal of 1:4. Further tandem mass spectrometry analysis of one of the modified peptides (Pro-Thr-His-Ile-Lys-Trp-Gly-Asp) strongly suggested binding of one pentanal molecule to the amino terminal proline and three pentanal molecules bound to the lysine residue. The latter species is suggested to be the actual fluorophore, through the formation of conjugated double bonds, and a possible reaction pathway through a combination of aldol condensation of pentanal and Schiff base formation with the lysine is suggested.

KEYWORDS: Carbonyl modifications; Schiff base formation; aldol condensation; peptides; fluorescence spectroscopy; MALDI TOF MS-MS; fluorophore; lipid oxidation

INTRODUCTION

Storage as well as processing influence the quality of food, and off-flavor is a consequence in many foodstuffs containing complex mixtures of fat and protein, such as milk and milk-derived products. Because of its impact on off-flavor, lipid oxidation has been intensively studied. The oxidation, however, can have both positive and negative effects on food quality (*1*). Carbonyl compounds are known to be formed as secondary products during peroxidation of unsaturated fatty acids (*2*−*4*), and carbonyls have been suggested to be of major importance in off-flavor formation (*5*−*7*). However, lipid-derived aldehydes have been found to form carbonyl adducts by binding of aldehydes to different amino acid side chains in proteins (*8*). These carbonyl adducts have been characterized by fluorescence spectroscopy, and the carbonyl adducts formed between saturated (*9*, *10*) or unsaturated (*11*) aldehydes and proteins result in the formation of fluorophores.

Condensation reactions between primary amino groups and aldehydes are well-known to form Schiff bases. In model systems, lysine (*12*) and methylamine (*13*) have been shown to form fluorophores after reaction with saturated aldehydes, indicating that Schiff bases may take part in the formation of fluorophores, but the structures of the adducts are not known in detail. Furthermore, the ability of saturated aldehydes to undergo aldol condensation (*14*) and thereby result in formation of unsaturated aldehydes complicates the reaction mechanism involved in the formation of the fluorophores between proteins and saturated aldehydes.

The objective of the present study was to study the fluorophore formed after reaction between pentanal and two different oligopeptides in order to obtain information on the formation and structure of the formed fluorophores. As Schiff bases are formed between aldehydes and primary amino groups, lysine-containing oligopeptides were applied for investigation of the reactions between the pentanal and the ϵ-amino group of lysine. Fluorescence spectroscopy was applied for characterization of the fluorophore formed between the pentanal and the peptide, and the structure of the formed adducts was further characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) MS/MS and gas chromatography (GC)-MS was applied for following aldol self-condensation of pentanal.

MATERIALS AND METHODS

Pentanal (>99%), α-cyano-4-hydroxy-cinnamic acid, and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich Chemie GmBh (Steinheim, Germany). Poros 50 R2 reverse phase column material was obtained from PerSeptive Biosystems (Framingham, MA). Sodium dihydrogen phosphate was obtained from Merck (Damstadt, Germany). Sodium monohydrogen phosphate was purchased from Baker Analyzed (Deventer, Holland). Acetonitrile (grade S) was purchased from Rathburn Chemicals Ltd. Walkerburn (Scotland). The oligopeptides Pro-

* To whom correspondence should be addressed. Tel: +4589991171. Fax: +4589991564. E-mail: LotteBach.Larsen@agrsci.dk.

10.1021/jf060003g CCC: $33.50
Published on Web 07/21/2006

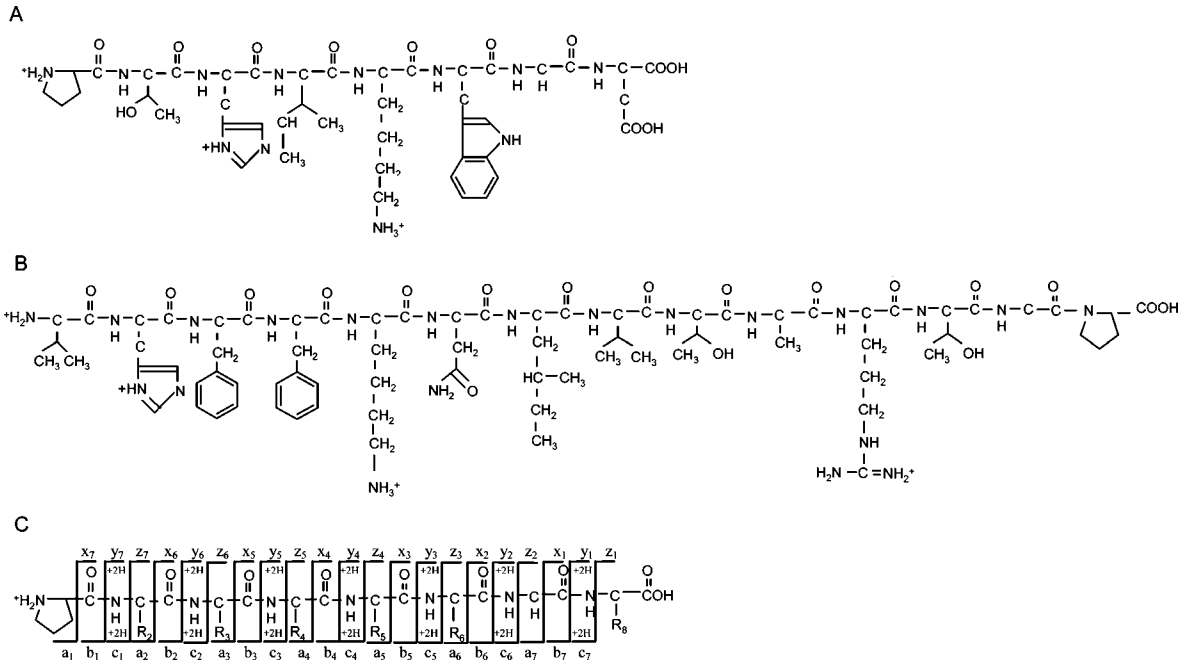


Figure 1. Primary protein structures of the peptides PTHIKWGD (**A**) and VHFFKNIVTARTP (**B**) and the fragmentation pattern (*17*) of PTHIKWGD (**C**) where R2, R3, R4, R5, R6, and R8 correspond to the side chain of threonine, histidine isoleucin, lysine, tryptophan, and aspartic acid, respectively. The b and y ions correspond to fragmentation at the peptide bond from the amino terminal proline and carboxyl terminal aspartic acid, respectively. b, a, and y ions are normally the dominating ions in a MS-MS spectra.

Thr-His-Ile-Lys-Trp-Gly-Asp (PTHIKWGD) and Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Ala-Arg-Thr-Pro (VHFFKNIVTARTP) were purchased from Bachem Holding AG (Bubendorf, Switzerland). Pentanal was dissolved in ethanol (96% v/v) Danish Distillers (Copenhagen, Denmark) prior to addition of buffer and peptide.

Fluorescence Spectroscopy. Fluorescence spectroscopy was applied for measurement of changes in fluorescence intensities after reaction of the two lysine-containing model peptides, PTHIKWGD and VHFFKNIVTARTP, respectively, and pentanal. Pentanal was dissolved in ethanol and gently mixed for 2 min before addition to a 3 mL quartz cell with a light path of 1 cm. The peptides were dissolved in 50 mM phosphate at pH 6.8 prior to addition to the pentanal solution in the quartz cell under gentle stirring. The final concentration of the peptides was 1 mg/mL, and the final concentration of ethanol was 10% in all experiments. All solutions had a temperature of 37 °C, and the quartz cell was thermostated to 37 °C during the experiments. The fluorescence method for detection of conjugated Schiff bases initially described by Trombly and Tappel (*15*) was used with the following modifications: Excitation was performed at 345 nm, and emission spectra were obtained from 370 to 500 nm using a LS 50B spectrofluorometer from Perkin-Elmer (Beaconsfield, England). Excitation was performed with a Xenon discharge lamp, and a photomultiplier with a modified S5 response measured emission. The bandwidth was 5 nm. The first emission spectrum was obtained approximately 1 min after addition of the peptide to the pentanal solution. Emission spectra were obtained over a period of approximately 1100 min. The rates of increase in emission intensities were calculated for the linear part of the sigmoid curves.

Mass Spectrometry (MS). Mass spectra were obtained on a MALDI Tandem TOF Mass Spectrometer, Ultraflex TOF-TOF from Bruker Daltonics GmbH (Bremen, Germany). All spectra were obtained in positive reflector mode using an accelerating voltage of 20 kV. All samples were incubated at 37 °C for approximately 24 h and measured as a function of incubation time. Custom-made chromatographic columns were used for concentration and desalting of the peptide solution prior to mass spectrometric analysis (*16*). A column consisting of 100−300 nL of Poros 50 R2 was equilibrated with 20 μL of 0.1% TFA before 5 μL of sample was accumulated at the column. The column was washed with 50 μL of 0.1% TFA and finally eluted directly on the MALDI target with a solution of saturated α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% TFA and dried before measurement. Tandem MS (MALDI TOF-TOF) was used for investigation of the species formed during incubation of the two model peptides with pentanal. FlexAnalysis version 2.4 and BioTools version 3.0 (Launch Bruker Daltonics, Germany) were applied for analysis of the mass spectra. The primary structures of the two used oligopeptides PTHIKWGD and VHFFKNIVTARTP are given in **Figure 1A**,**B**, respectively. Furthermore, the fragmentation pattern of peptides subjected to tandem MS first proposed by Roepstorff and Fohlman (*17*) is given in **Figure 1C**.

GC-MS. A 100 mM concentration of pentanal was dissolved in 96% ethanol before dilution into phosphate buffer, pH 6.8, resulting in 10% ethanol in the final sample that was measured using headspace GC-MS. A 100 μL amount of sample was prepared in a 12.5 mL vial and sealed with Teflon before incubation at 37 °C. The headspace was analyzed for volatile compounds using a StableFlex Divinylbenzene/Carboxen/PDMS solid phase microextraction (SPME) fiber with a film thickness of 30 μm from Supelco (Bellefonte, PA), which was incubated for 2 min in the headspace of each sample. Desorption of the sample from the fiber was performed into the inlet of a STAR 3400cx Varian GC (Walnut Creek, CA) that was equipped with a 122-3232, DB FFAP (J&W Scientific, Folsom, CA) with the dimensions 0.25 mm i.d., 0.25 μm silica film, 30 m. Helium was used as the carrier gas with a constant pressure of 103461 N/m^2 through the column. The 1078 split/splitless injector was kept at 250 °C. A Restek open low volume liner with an inner diameter of 0.75 was applied. The column temperature was programmed from 35 to 100 °C with a rate of 10 °C/min and from 100 to 240 °C with a rate of 3.5 °C/min. Mass spectral analysis was performed in the mass range 50−3500 *m*/*z* on a SATURN 2000 Iontrap Varian MS with a temperature of 200 °C and a fragmentation voltage of 70 eV. The temperature of the manifold was 60 °C. Integration of the peak that corresponded to a reaction product between two pentanal molecules with the elimination of water was performed by SaturnView Version 5.52 from Varian. Tentative identification of the compound was performed applying NIST Mass Spectral Search Program version 1.7.

RESULTS

The formation of fluorescence products was studied over time during incubation of the oligopeptides with different concentrations of pentanal. The excitation and emission spectra for the

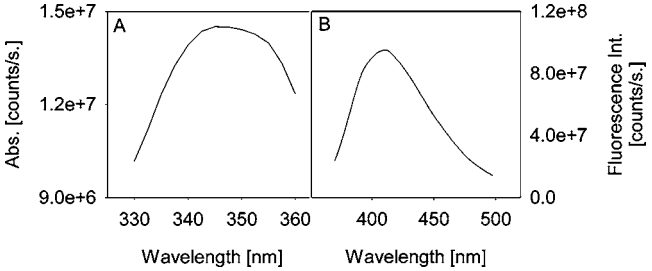


Figure 2. Excitation (**A**) and emission spectra (**B**) obtained for the peptide PTHIKWGD after 24 h of incubation with pentanal at a molar ratio of 1:100 at 37 °C and pH 6.8.

peptide PTHIKWGD are shown in **Figure 2A**,**B**, respectively. In aqueous solution at pH 6.8 and in the presence of 10% of ethanol, an excitation maximum was observed between 340 and 350 nm for both oligopeptides, while the emission spectra had maxima between 410 and 420 nm, corresponding to blue fluorescent light. A kinetic study was carried out with the duration of 1100 min, where emission spectra were collected every 100 min for peptide PTHIKWGD after incubation of different molar ratios of peptide:pentanal (1:100, 1:75, 1:50, and 1:10). The development in fluorescence after incubation of the peptide PTHIKWGD (1 mg/mL) in the presence of 100-fold molar excess of pentanal is shown in **Figure 3A**. Increments in fluorescence intensities at 415 nm calculated from the emission spectra in **Figure 3A** showed a sigmoid curvature (**Figure 3B**). Sigmoid curvatures were also seen for PTHIKWGD incubated with 10, 50, and 75 molar excess of pentanal (**Figure 3B**). Similar results were found for the reaction of VHFFKNIVTARTP incubated with 100-fold molar excess of pentanal (data not shown). The rate of the reaction between aldehyde and peptide was determined as the linear part of the sigmoid curves, as indicated by the line in **Figure 3B** to describe the rate of fluorescence development. These reaction rates were found to follow first-order kinetics with regard to the concentration of pentanal (**Figure 3C**).

The reaction products formed in the reaction between the oligopeptides and the pentanal were further studied by MALDI TOF MS to gain information on the masses of the reaction products. Apart from the mass of the unmodified model peptide of 953 *m*/*z* for PTHIKWGD, four further mass peaks at 1021, 1089, 1155, and 1223 *m*/*z* were seen in the mass spectrum (**Figure 4A**). The expected masses of reaction products for binding one, two, three, or four molecules of pentanal would be 1021, 1089, 1157, and 1225, respectively, with steps of 68 *m*/*z*. This mass of 68 corresponds to the mass of bound pentanal, with an original mass of 86, after Schiff base condensation reaction with peptide leading to loss of 18 *m*/*z* due to splitting of water. The ions corresponding to binding of three (1157 *m*/*z*) or four (1225 *m*/*z*) pentanal molecules to the peptide were, however, not the dominant ion masses in the spectrum. Instead, two prevalent ions with *m*/*z* of 1155 and 1223 were detected, and it is proposed that these ions reflect binding of three and four pentanal molecules, respectively, to the peptide, where two hydrogens were eliminated. The same elimination of two hydrogen atoms was also seen for the peptide VHFFKNIVTARTP (original *m*/*z* 1529) after binding of three (1732 *m*/*z*) and four (1800 *m*/*z*) pentanal molecules (**Figure 4B**). Furthermore, for peptide VHFFKNIVTARTP, the modified forms with one or two attached molecules of pentanal were also seen, corresponding to *m*/*z* values of 1598 and 1666 (**Figure 4B**). Further ion masses were seen in both mass spectra, and they were all found to correspond to addition of oxygen or sodium. As no ion masses were observed between 1900 and 2450 (*m*/*z*) for PTHIKWGD or between 3000 and 3600 (*m*/*z*) for VHFFKNIVTARTP, neither of the two peptides seemed to form intermolecular cross-linkages after incubation with pentanal.

The dominating ions (953, 1021, 1155, and 1223 *m*/*z*) that were identified by MALDI TOF for peptide PTHIKWGD were further analyzed by MALDI TOF-TOF to identify the positions of the modifications with pentanal (**Figure 5A**−**D**). A MS-MS spectrum of the unmodified peptide is given in **Figure 5A**, with the most dominant ion masses being identified as b ions and a ions according to the fragmentation pathway given in **Figure 1C**. The ion masses of the b ions and their corresponding a ions are given to the right of the spectra. According to a shift of 68 (*m*/*z*) in b ions and their corresponding a ions, it was shown that the specific binding of one pentanal molecule to the peptide (*m*/*z* 1021) was initially obtained at the amino-terminal proline residue of the peptide PTHIKWGD (**Figure 5B**). Analysis of the ion *m*/*z* 1155 indicated that the dominant species was the peptide modified by binding of one pentanal molecule at the amino terminal proline residue and two molecules of pentanal attached to lysine (**Figure 5C**). B ions from a species with three pentanal molecules bound at the lysine residue were also seen, but sequencing of this species could, however, not be obtained further than to the lysine residue, and the intensities of the ions were rather limited (ions not indicated). Sequencing of the species with one pentanal molecule bound at the amino terminal proline and two at the lysine residue could be performed throughout the whole sequence, thus indicating this to be the superior species of the ion with *m*/*z* of 1155. Analysis of the fragmentation pattern of the ion (1223 *m*/*z*)

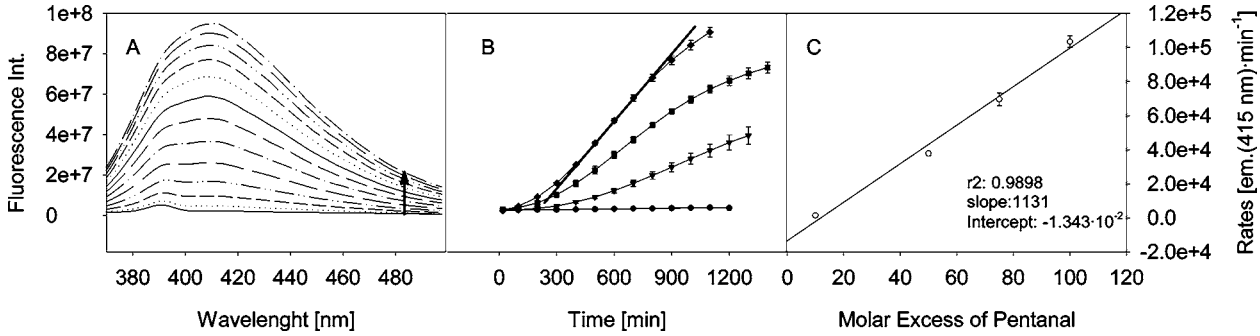


Figure 3. (**A**) Emission spectra obtained over time with 100 min interval for 1 mg/mL PTHIKWGD incubated at pH 6.8 with a molar excess of pentanal (1:100) after excitation at 345 nm. Time is indicated by an arrow. (**B**) Emission intensities obtained at 415 nm increased over time and showed a sigmoid curved for molar excess of pentanal ◆ (1:100), ■ (1:75), ▼ (1:50), and ● (1:10), respectively (*y*-axis as in **A**). (**C**) Dependence of pentanal concentration obtained as rates from the linear part of the sigmoid curves as indicated in **B**. The measurements were carried out in triplicate. Standard derivations are indicated as bars.

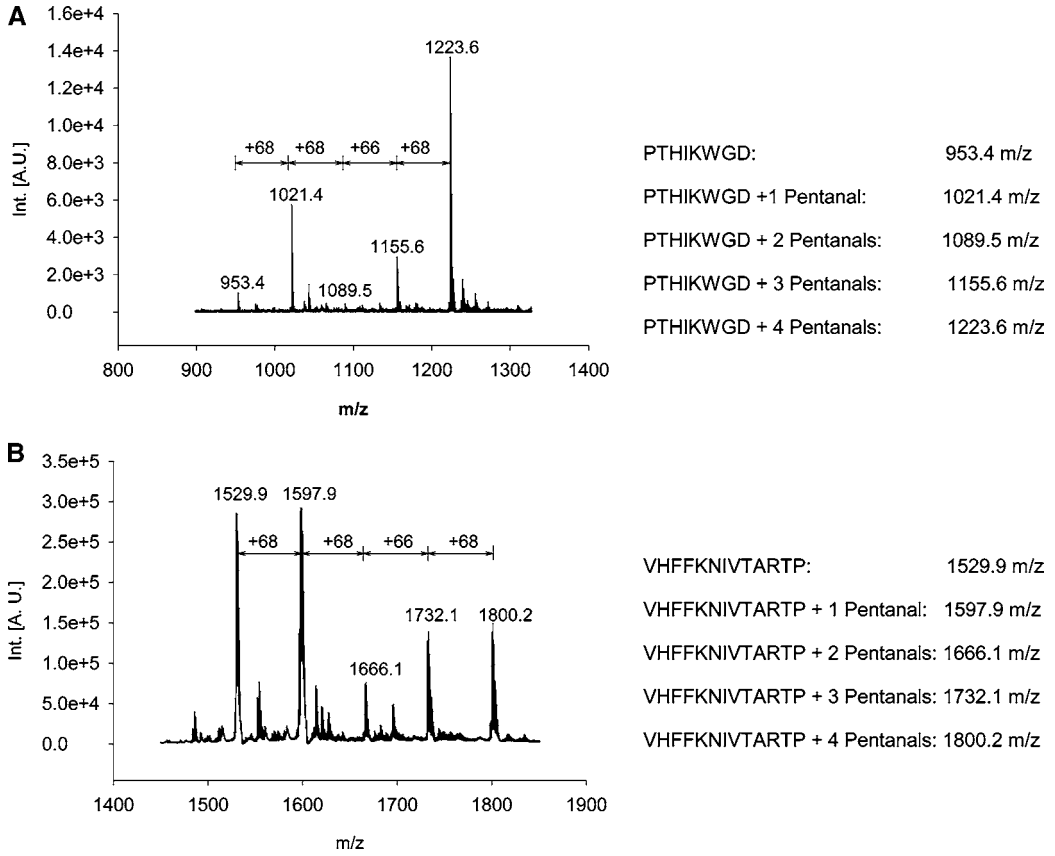


Figure 4. MALDI TOF mass spectra of (**A**) 1 mg/mL PTHIKWGD and (**B**) VHFFKNIVTARTP after incubation with pentanal in the molar ratio of 1:100 for 24 h at pH 6.8. The ion masses in **A** correspond to the unmodified PTHIKWGD (953 *m*/*z*) and the peptide with one (1021 *m*/*z*), two (1089 *m*/*z*), three (1155 *m*/*z*), and four (1223 *m*/*z*) pentanal molecules bound to it. In **B**, the ion masses at 1530 (*m*/*z*), 1598 (*m*/*z*), 1666 (*m*/*z*), 1732 (*m*/*z*), and 1800 (*m*/*z*) correspond to the peptide mass of VHFFKNIVTARTP and the peptide modified with one, two, three, and four pentanal molecules, respectively.

indicated that the first of the four pentanal molecules was located at the amino-terminal proline and the last three were bound to the lysine residue (**Figure 5D**). The most dominating ion mass (1084 *m*/*z*) in the MS-MS spectrum could unfortunately not be identified, neither as b, a, nor y ion.

The relative intensities of the ions corresponding to addition of 1−4 pentanal molecules to peptide PTHIKWGD (with *m*/*z* values of 1021, 1089, 1155, and 1223, respectively) obtained by MALDI TOF were followed over time in an attempt to describe the development of the identified peptide modifications (**Figure 6**). It is well-known that peak intensities of different ions are not quantitatively comparable in MALDI TOF due to differences in ionizations of different ions. Therefore, the relative ion intensities were calculated at each time point, which permits analysis of the development in the amount of each ion, provided that each of the individual ions ionizes at the same level at each time point. The data therefore can be used in interpretation of the development in the prevalence of each ion but cannot be used for comparison between different ions. According to this analysis, the intensity of the ion describing binding of one pentanal to the amino terminal proline (*m*/*z* 1021) was formed instantaneously and declined very quickly. The ion, which corresponds to the binding of two pentanal molecules (*m*/*z* of 1089), was low in intensity at all times. In contrast, the ion indicating binding of three pentanal molecules (*m*/*z* of 1155) was rather intensive at the beginning of the analysis, and the intensity declined within the first 200 min, then it increased somewhat before it became rather constant. The ion corresponding to binding of four pentanal molecules (*m*/*z* of 1223) showed an opposite progress as compared to the ion with three pentanal additions, thus increasing for the initial 120−200 min. These findings indicate that the peptide species with one pentanal molecule attached to the amino-terminal proline residue and two additional pentanal molecules bound to the lysine residue binds a further pentanal molecule. This reaction results in a product with three pentanal molecules bound to the ϵ-amino group of lysine plus one pentanal molecule bound at the amino-terminal proline.

As MS measurements indicated binding of up to four pentanal molecules per peptide molecule, and TOF-TOF measurements indicated that three pentanal molecules were located at the lysine residue of the peptide PTHIKWGD, it was of interest to study whether pentanal itself underwent any self-condensation reaction before reaction with the lysine residue of the peptide. GC-MS was therefore applied for investigation of the possibility of aldol condensation reaction of pentanal to occur. The results of the GC-MS investigation indicated that pentanal itself went through aldol condensation. This was suggested from the molecular ion mass of 154 *m*/*z*, and searching the NIST library indicated that pentanal self-condensation resulted in the formation of 2-propyl-2-heptenal. The concentration of this compound increased during incubation at 37 °C and pH 6.8 for 1200 min (**Figure 7**). The structure of the aldol condensation product includes two reactive sites, an aldehyde and a double bond, making more reactions and cross-linkages possible. Neither of the ion masses seen by MALDI TOF did, however, indicate introduction of peptide cross-linkages after reaction with pentanal.

DISCUSSION

The emission maximum was identified at 415 nm after excitation at 345 nm, and the location of the excitation maximum between 340 and 350 nm was identified after incubation of

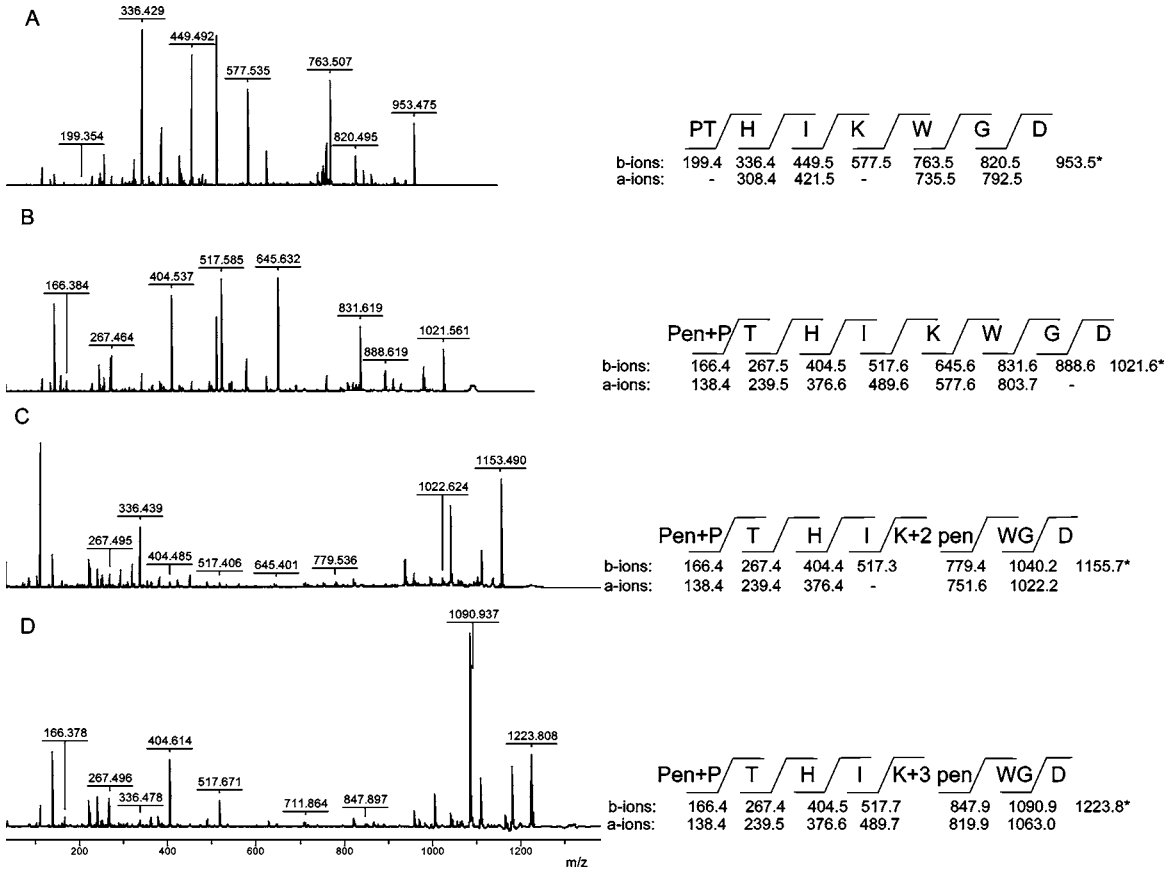


Figure 5. MALDI TOF-TOF performed on the peaks obtained by MALDI TOF in **Figure 4**. Only the b ions are indicated in the spectra at the left of the figure. Amino acids single letter codes are given for the sequence of PTHIKWGD at the right part of the figure with masses corresponding to the a and b ions found in the MS-MS spectra. Binding of pentanal is indicated as "pen". a and b ions are indicated according to the fragmentation pattern first suggested by Roepstorff and Fohlman (*17*). (**A**) Unmodified peptide (PTHIKWGD) with a molecular mass of *m*/*z* 953. (**B**) Binding of one pentanal molecule to PTHIKWGD corresponding to a molecular mass of *m*/*z* 1021. (**C**) Binding of three pentanal molecules to PTHIKWGD corresponding to a molecular mass of 1155 (*m*/*z*). (**D**) Binding of four pentanal molecules to PTHIKWGD corresponding to a molecular mass of 1223 (*m*/*z*).

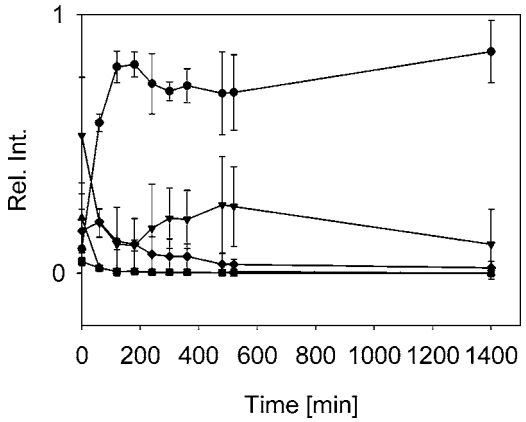


Figure 6. Relative ion intensities after binding of ● (4), ▼ (3), ■ (2), ◆ (1), and ▲ (0) pentanal molecules to the oligopeptide PTHIKWGD as measured by MALDI TOF after incubation of 1 mg/mL peptide with a molar excess of pentanal (1:100) at pH 6.8 and 37 °C. The amount of each ion species was calculated as relative peak intensities at each time point. The measurements were performed as four true replicates, and standard derivations are given as bars.

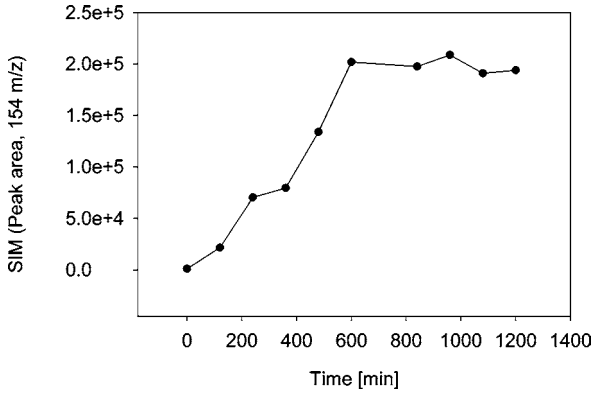


Figure 7. Aldol condensation of pentanal measured with headspace GC-MS over time. A 100 mM concentration of pentanal was incubated at pH 6.8 and 37 °C over a period of 0–1200 min. The increment in the area of the molecular ion (154 *m*/*z*), whose mass corresponds to reaction between two pentanal molecules with elimination of water, was followed with a 2 h interval for the period 0–1200 min. SIM, selected ion monitoring.

pentanal with either of the two model peptides PTHIKWGD or VHFFKNIVTARTP in the presence of 10% ethanol at pH 6.8. These findings are in agreement with earlier reported results after reaction between ϵ-amino groups of lysines and pentanal in 80% methanol at pH 7.0 (*13*). The fluorescence spectroscopy measurements revealed first-order kinetics for the reaction between the peptide (PTHIKWGD) and the pentanal in relation to the concentration of pentanal. The linear dependence on the concentration of pentanal illustrates that fluorescence spectroscopy is a suitable method for the detection of modification of peptides with pentanal or pentanal-derived products. The fluorescence spectroscopy is, however, unspecific, and the particular reaction products should therefore be identified by other means, e.g., by comparison of fluorescence spectroscopy

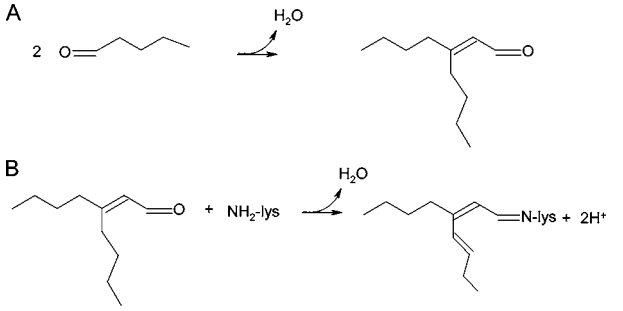


Figure 8. (**A**) Schematic representation of reaction product formed by aldol condensation of two pentanal molecules. (**B**) Suggested structure of the peptide species formed as a result of Schiff base formation between the lysine and the product obtained from the aldol condensation reaction in **A**. The elimination of two protons is suggested to occur and being introduced in conjugation with the others to obtain the highest structural stability of the molecule.

data with MS results. The MALDI TOF analysis indicated that the reaction between the amino-terminal proline of PTHIKWGD and one pentanal molecule was obtained almost instantaneously. The observed ion mass was in agreement with a condensation reaction with increments of 68 *m*/*z*, and thus, a Schiff base is suggested to have been formed between the amino-terminal proline of PTHIKWGD and the one pentanal molecule. Binding of pentanal to the amino terminus prior to binding to the ϵ-amino group at the lysine residue is in agreement with the findings of Stapelfeldt and Skibsted (*12*). No appreciable changes were observed in the fluorescence intensities during the first 100 min; therefore, binding of one pentanal molecule at the amino terminal proline is not believed to contribute to the increase in fluorescence intensities observed later in the kinetic study. A more likely fluorophore includes the aldol condensation product of two pentanal molecules, 2-propyl-2-heptenal, due to the presence of conjugated double bonds (**Figure 8A**). This condensation product formed in the pentanal solution was identified by headspace GC-MS. Thus, the formation of the oligopeptide species with two or three pentanal molecules bound to the lysine residue results in formation of potential fluorophores. An aldol condensation reaction has earlier been observed in a study with 1-butanal and polylysine (*8*), and it was reported that the reaction between the polylysine and the aldol condensation product 2-ethyl-2-hexenal yielded higher fluorescence intensities than the reaction between polylysine and butanal. Cross-linkages in protein structures have been proposed to be due to reactions between the protein amino groups and the butanal and its aldol condensation product, 2-ethyl-2-hexenal (*9*).

The finding that the pentanal solution can undergo aldol condensation reaction, resulting in the formation of 2-propyl-2-heptenal, makes more reactions in the polypeptide side chains possible, due to the presence of both an aldehyde group as well as of a double bond in the condensation product. Michael addition was found after lysine reaction with 2-pentenal and 2-hexenal (*18*), and as mentioned above, Schiff base formation is possible between lysine and any aldehyde. Michael addition should, however, yield an increase in ion mass of 154 as compared with an increase in the ion mass of 136 in Schiff base formation due to the loss of water (18 *m*/*z*) in the Schiff base formation, which is not lost by Michael addition. No peaks were seen corresponding to a mass increase of 154 *m*/*z*, and as no further cross-linking was detected by MALDI TOF, Michael addition is not believed to give a rise to fluorescence in the system applied in this study. The lysine residue was, however, strongly modified, and it is believed that the observed 2-alkenal-(2-propyl-2-heptenal) and the ϵ-amino group of lysine react through the formation of a Schiff base.

As mentioned, Schiff base formation between the formed 2-alkenal would be expected to yield an increase in mass of 136. The predominant ion mass corresponds, however, to an increase of 134 *m*/*z*, hence suggesting elimination of two hydrogen atoms. Schiff base formation between 2-alkenal, 2-propyl-2-heptenal, and amino groups was suggested to be the initial reaction followed by an oxidation with the formation of the ring structure and loss of two protons (*19*). Pyridinium adducts were further suggested to be the primary ring structure in modification of primary amines with 2-alkenals in the ratio 1:3 (*19*), which agrees with the higher stability of a ring structure as compared with an aliphatic chain. Formation of pyridinium salt was, however, not identified as fluorophore in a system including butanal and methylamine (*13*). In this study, still two hydrogen atoms were eliminated according to the MS results. We propose the elimination of two hydrogen atoms to be due to introduction of a double bond, when 2-propyl-2-heptenal was bound to the ϵ-amino group of lysine. The molecule will obtain the highest structural stability if a double bond was introduced in conjugation with the other two; therefore, a possible structure of the dialkylated lysine is suggested to be as shown in **Figure 8B**. It would be interesting, in future studies, to solve the exact structures of these adducts with additions of either three or four pentanal molecules by, e.g., NMR. This would require preparative isolation of the different adducts. An attempt of separation of PTHIKWGD + three molecules of pentanal and PTHIKWGD + four molecules of pentanal by HPLC was carried out in this study, but it was not possible, in our hands, to achieve separation of these adducts (results not shown).

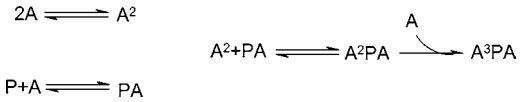
The relative ratio of the intensities obtained from the MALDI TOF in the present study, however, illustrates that the only ion increasing in intensity during the entire incubation period was the one related to the peptide modified with four pentanal molecules: three pentanal molecules bound to the lysine residue and one at the amino terminal proline, hence indicating this could be the adduct responsible for the fluorescence detected. An earlier study described that both Phe1 and Lys29 of the B chain of insulin were dialkylated by hexanal adducts (*20*). The dialkylation of the lysine residue of insulin B chain in that study agrees with the binding of 2-propyl-2-heptenal representing a dialkylation of the lysine residue. The amino terminal proline of PTHIKWGD was, however, only modified with one pentanal molecule and the lysine with up to three pentanal molecules. Besides binding of the aldol condensation product, one additional pentanal was found to bind to the lysine residue. Apart from condensation of two molecules of pentanal, as shown by GC-MS, no higher pentanal condensation adducts were detected by additional investigations using GC-MS with liquid injection (results not shown). This indicates that the third pentanal molecule binds either to the unmodified lysine ϵ-amino group, followed by binding to the aldol condensation adduct, or to the already formed 2-propyl-2-heptenal-modified lysine residue. The loss of two hydrogen atoms was observed for both the ion masses corresponding to binding of three and of four pentanal molecules. These results indicate that the third pentanal is able to bind the ϵ-amino group, which already has been modified by the 2-alkenal. As the mass peak, which corresponds to binding of one pentanal molecule at the lysine residue (*m*/*z* 1089, assuming that one is already bound at the amino terminal proline), was almost nonexistent, the following reaction mechanism is suggested

$$A + P \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} PA \quad (1)$$

$$A + A \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} A^2 \quad (2)$$

$$A^2 + PA \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} A^2PA \quad (3)$$

$$A + A^2PA \underset{k_4}{\rightleftharpoons} A^3PA \quad (4)$$



where A is pentanal, P is the peptide, PA is the amino terminal bound to one pentanal molecule, A^2 is the aldol condensation product, A^2PA is the peptide modified with the aldol condensation product at the lysine residue and one pentanal molecule bound to the amino terminal proline, and A^3PA is the peptide modified with a third pentanal molecule at the lysine residue.

Taken together, it is suggested that the species A^3PA (the peptide modified with three pentanal molecules bound at the lysine residue and one at the amino terminal proline) is the fluorophore of the model systems applied in this study. Application of fluorescence spectroscopy for the detection of Schiff bases in food systems may therefore not reflect a direct reaction between saturated aldehydes and proteins/peptides but rather be due to products as a result of a combination of aldol condensation and Schiff base formation.

ABBREVIATIONS USED

GC, gas chromatography; MALDI TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; PTHIKWGD, NH_2-proline-threonine-histidine-isoleucine-lysine-tryptophan-glycine-aspartic acid-OH; SPME, solid phase microextraction; TFA, trifluoroacetic acid; VHFFKNIVTARTP, NH_2-valine-histidine-phenylalanine-phenylalanine-lysine-asparagine-isoleucine-valine-threonine-alanine-arginine-threonine-proline-OH.

ACKNOWLEDGMENT

We thank Dr. Torben Ellebæk Petersen and Dr. Christian W. Heegaard, Protein Chemistry Laboratory, University of Aarhus, Denmark, and Dr. Henrik Stapelfeldt, CP Kelco, Denmark, for inspiring discussions. The suggestions and comments of five anonymous referees are greatly appreciated.

LITERATURE CITED

(1) Zamora, R.; Hidalgo, F. J. Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 49−59.

(2) Forss, D. A.; Pont, E. G.; Stark, W. The volatile compounds associated with oxidized flavour in skim milk. *J. Dairy Res.* **1955**, *22*, 91−102.

(3) Kim, Y. D.; Morr, C. V. Dynamic headspace analysis of light activated flavor in milk. *Int. Dairy J.* **1996**, *6*, 185−193.

(4) Esterbauer, H.; Jurgens, G.; Quehenberger, O.; Koller, E. Autoxidation of human low-density-lipoprotein—Loss of polyunsaturated fatty-acids and vitamin-e and generation of aldehydes. *J. Lipid Res.* **1987**, *28*, 495−509.

(5) Bendall, J. G.; Olney, S. D. Hept-*cis*-4-enal: Analysis and flavour contribution to fresh milk. *Int. Dairy J.* **2001**, *11*, 855−864.

(6) Sattar, A.; deMan, J. M. Photooxidation of milk and milk product: A review. *Crit. Rev. Food Sci. Nutr.* **1975**, *7*, 13−37.

(7) Bhat, G. S.; Murthy, M. K. R.; Rao, M. B. Production of carbonyls during spontaneous oxidation of cow and buffalo milks. *J. Food Sci. Technol.-Mysore* **1981**, *18*, 210−211.

(8) Refsgaard, H. H. F.; Tsai, L.; Stadtman, E. R. Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 611−616.

(9) Kikugawa, K.; Iwata, A.; Beppu, M. Formation of cross-links and fluorescence in polylysine, soluble-proteins and membrane-proteins by reaction with 1-butanal. *Chem. Pharm. Bull.* **1988**, *36*, 685−692.

(10) Stapelfeldt, H.; Skibsted, L. H. Modification of beta-lactoglobulin by aliphatic-aldehydes in aqueous-solution. *J. Dairy Res.* **1994**, *61*, 209−219.

(11) Yamaki, S.; Kato, T.; Kikugawa, K. Characteristics of fluorescence formed by the reaction of proteins with unsaturated aldehydes, possible degradation products of lipid radicals. *Chem. Pharm. Bull.* **1992**, *40*, 2138−2142.

(12) Stapelfeldt, H.; Skibsted, L. H. Kinetics of formation of fluorescent products from hexanal and L-lysine in a two-phase system. *Lipids* **1996**, *31*, 1125−1132.

(13) Kikugawa, K.; Sawamura, A. Formation of fluorescent substances in reaction of aliphatic-aldehydes and methylamine. *J. Am. Oil Chem. Soc.* **1987**, *64*, 1156−1162.

(14) Suyama, K.; Arakawa, T.; Adachi, S. Free fatty aldehydes and their aldol condensation products in heated meat. *J. Agric. Food Chem.* **1981**, *29*, 875−878.

(15) Trombly, R.; Tappel, A. Fractionation and analysis of fluorescent products of lipid peroxidation. *Lipids* **1975**, *10*, 441−447.

(16) Gobom, J.; Nordhoff, E.; Mirgorodskaya, E.; Ekman, R.; Roepstorff, P. Sample purification and preparation technique based on nanoscale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrosc.* **1999**, *34*, 105−116.

(17) Roepstorff, P.; Fohlman, J. Proposal for a common nomenclature for sequence ions in mass-spectra of peptides. *Biomed. Mass Spectrosc.* **1984**, *11*, 601.

(18) Ichihashi, K.; Osawa, T.; Toyokuni, S.; Uchida, K. Endogenous formation of protein adducts with carcinogenic aldehydes—Implications for oxidative stress. *J. Biol. Cehm.* **2001**, *276*, 23903−23913.

(19) Baker, A.; Zidek, L.; Wiesler, D.; Chmelik, J.; Pagel, M.; Novotny, M. V. Reaction of *N*-acetylglycyllysine methyl ester with 2-alkenals: An alternative model for covalent modification of proteins. *Chem. Res. Toxicol.* **1998**, *11*, 730−740.

(20) Fenaille, F.; Tabet, J. C.; Guy, P. A. Study of peptides containing modified lysine residues by tandem mass spectrometry: Precursor ion scanning of hexanal-modified peptides. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 67−76.

Received for review January 2, 2006. Revised manuscript received June 12, 2006. Accepted June 18, 2006.

JF060003G