JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Binding of Flavor Compounds and Whey Protein Isolate as Affected by Heat and High Pressure Treatments

JANINA KÜHN,[†,‡] THÉRÈSE CONSIDINE,[§] AND HARJINDER SINGH*[,†]

Riddet Centre, Massey University, Private Bag 11 222, Palmerston North, New Zealand, Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11 222, Palmerston North, New Zealand, Fonterra Research Centre, Private Bag 11 029, Palmerston North, New Zealand

The interactions of whey protein isolate (WPI) and flavor compounds (2-nonanone, 1-nonanal, and *trans*-2-nonenal) were investigated, and the influence of flavor compound structure and heat and high pressure denaturation on the interactions were determined by using headspace solid-phase microextraction (SPME) and gas chromatography (GC). The binding of WPI and the flavor compounds decreased in the order *trans*-2-nonenal > 1-nonanal > 2-nonanone. The differences in binding can be explained with hydrophobic interactions only in the case of 2-nonanone, whereas the aldehydes, in particular *trans*-2-nonenal, can also react covalently. Heat and high pressure treatment affected protein−flavor interactions depending on the structure of the flavor compound. Upon both heat and high pressure denaturation, the binding of 2-nonanone to WPI decreased, while the binding of 1-nonanal remained unchanged, and the affinity for *trans*-2-nonenal increased rapidly. The results suggest that hydrophobic interactions are weakened upon heat or high pressure denaturation, whereas covalent interactions are enhanced.

KEYWORDS: Whey protein isolate; 2-nonanone; 1-nonanal; *trans*-2-nonenal; flavor binding; heat treatment; high pressure treatment; solid-phase microextraction

INTRODUCTION

The binding of flavor compounds to proteins can result in an enormous change in perceived flavor intensities, especially in reduced-fat products (*1*). Milk proteins are naturally present and added to a variety of food products and thus may change overall flavor profiles of foods by binding flavor compounds. Whey proteins have been found to have a higher flavor binding capacity than caseins (*2, 3*).

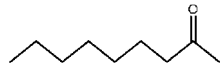
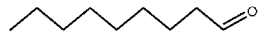
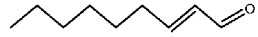
Both reversible binding and irreversible binding can occur between proteins and flavor compounds. The type of interaction between a protein and a flavor compound depends on the nature of both the protein and the aroma compound. As most aroma compounds are hydrophobic in nature, hydrophobic and reversible binding is predominant (*4*). On the other hand, certain flavor compounds, such as aldehydes, can form covalent bonds with proteins (*5*). These include amide and ester formation, the condensation of aldehydes with amino groups ("Schiff base" formation) and sulfydryl groups, and addition reactions with unsaturated flavor compounds ("Michael addition") (*5, 6*).

Milk protein−flavor interactions are very dependent on the conformational state of a protein. Therefore, factors such as pH, temperature, and high pressure that influence protein conformation can markedly change flavor binding characteristics of proteins (*3, 7*). Because heat treatment is an important step during the processing and preparation of many protein containing foods, the investigation of flavor binding to heat denatured proteins is of great importance. However, most studies have examined native proteins and their flavor binding behavior. In contrast to the caseins, whey proteins are susceptible to heat denaturation. In a previous study, it was found that β-lg is the whey protein mainly responsible for the binding of the model flavor compound 2-nonanone in WPI (*2*). Most flavor compounds are preferably bound in the hydrophobic pocket of β-lg (*8, 9*); however, a second high affinity binding site near the surface of the protein (*10*) and several weaker binding sites (*11, 12*) have also been suggested. Upon heat treatment, β-lg partially unfolds and aggregates via hydrophobic association and intra- and interprotein disulfide bonds (*13*). Among the limited number of studies on flavor binding to denatured whey proteins in the literature, many contradictions exist. Upon whey protein denaturation, decreases (*11, 14, 15*) as well as increases (*16*) in flavor binding have been reported. However, the use of different flavor compounds and different methodologies makes a comparison between studies difficult. Protein unfolding may increase flavor binding by revealing previously buried hydrophobic binding sites, or it may decrease the

* To whom correspondence should be addressed. E-mail: H.Singh@massey.ac.nz. Phone: +64-6-350-5545. Fax: +64-6-350-5655.
[†] Riddet Centre, Massey University.
[‡] Institute of Food, Nutrition and Human Health, Massey University.
[§] Fonterra Research Centre.

10.1021/jf801810b CCC: $40.75
Published on Web 10/21/2008

Table 1. Physicochemical Characteristics of the C9 Flavor Compounds

Flavor compound	Structure	M (g/mol)	Aqueous solubility (g/l) (25 °C)	logP [a)]
2-nonanone		142.24	0.4 (*25*)	2.9 (*26*)
1-nonanal		142.24	0.096 (*27*)	3.27 (*28*)
trans-2-nonenal		140.23	n.d.	3.06 (*28*)

[a)] Value of the logarithm of the partition coefficient between *n*-octanol and water.

[a] Value of the logarithm of the partition coefficient between *n*-octanol and water.

binding by modifying specific binding sites for flavor compounds. The aggregation of protein molecules may lead to a decrease in flavor binding due to hydrophobic protein−protein interactions instead of protein−flavor interactions. Clearly the mechanisms of binding to heat denatured proteins require further investigation.

As an alternative to thermal processing of food products, high pressure treatment is becoming increasingly important as it retains several desirable food quality attributes. Whey proteins have been found to be susceptible to high pressure treatment, which unfolds them and allows both noncovalent and disulfide intermolecular interactions to occur (*17*). Only two studies have been reported on the effect of high pressure treatment on protein−flavor interactions (*18, 19*); both indicated that high pressure treatment of whey proteins can decrease the binding affinity for flavor compounds or have no effect on the interactions, depending on the nature of the flavor compound.

The aim of this study was to compare the binding of three structurally similar flavor compounds, 2-nonanone, 1-nonanal, and *trans*-2-nonenal (**Table 1**), to WPI in aqueous solution and to investigate the effect of heat and high pressure treatment on the extent of protein−flavor binding, using an automated headspace SPME method followed by GC and flame ionization detection (FID).

MATERIALS AND METHODS

Materials. *Water.* Distilled, deionized water was obtained from a NANOpure II water purification system (Barnstead, Dubuque, IA).

Whey Protein Isolate. WPI (ALACEN 895) was obtained from Fonterra Co-operative Group Ltd., New Zealand. The WPI consisted of 93.3% protein (65% β-lg, 16% α-lactalbumin, 3% bovine serum albumin) 0.3% fat, and 4.6% moisture. A WPI solution (0.5%, w/v) was prepared in 0.1 M phosphate buffer (pH 7.2) and stored at 5 °C for 6 h to allow complete hydration.

Flavor Compounds. The flavor compounds were selected based on their structure to compare the effect of the position of the keto group and the presence of a double bond on protein−flavor interactions. 2-Nonanone, 1-nonanal, and *trans*-2-nonenal (**Table 1**) were purchased from Sigma-Aldrich (St. Louis, MO) and were of 99%, 95%, and 97% purity, respectively. Stock solutions of the flavor compounds (50 mg/kg) were prepared in water. An external standard calibration was used to calculate the extent of binding. Standards of each flavor compound were prepared by diluting the stock solution in buffer to obtain final flavor concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/kg. The amount of free (not protein bound flavor) [F] was estimated from the standard curve. The extent of flavor binding was estimated as follows, where [T] is the total amount of flavor (1.0 mg/kg): binding (%) = ([T] − [F]) × 100/[T].

Amino Acid Analysis. To determine the amino acids that react with *trans*-2-nonenal, equal volumes of WPI (0.05%) in water and *trans*-2-nonenal stock solution (50 mg/kg) were combined and equilibrated for 40 h at 5 °C. A control sample was prepared by combining equal volumes of WPI (0.05%) and water. The molar ratio of WPI:*trans*-2-nonenal was approximately 1:10. Amino acids in both samples were determined by hydrochloric acid hydrolysis (methionine and cysteine by performic acid oxidation) followed by HPLC separation using the AOAC official method 994.12 (*20*).

Heat Treatment. Samples were prepared by transferring 6.86 mL of WPI solution (0.5%) in 20 mL headspace vials (Chromacol, Herts, UK). For samples with flavor addition before heat treatment, 140 μL of the flavor stock solution (50 mg/kg) was added to obtain a final flavor concentration of 1.0 mg/kg. The vials were immediately sealed with silicone/PTFE septa and magnetic tin crimp caps (Chromacol, Herts, UK) and equilibrated for 40 h at 5 °C. Subsequently, the mixtures were heated for 0, 0.5, 1, 2, 5, 10, 20, 40, 60, and 80 min in a temperature-controlled water bath at 80 (±0.5) °C and immediately cooled in ice water for 5 min. All samples were manually agitated every 5 min during heat treatment. The sample vials were kept at room temperature for about 2 h before being analyzed by automated headspace SPME and GC-FID.

For samples with flavor added after heat treatment, the WPI solutions were first heated, and immediately after the samples were cooled down in ice water, 140 μL of flavor stock solution was added. After equilibration (40 h, 5 °C), they were analyzed by headspace SPME and GC-FID.

Controls were prepared in the same way as the samples with flavor added before heat treatment by weighing 6.86 mL of buffer in 20 mL headspace vials and adding 140 μL of flavor stock solution. Controls were not heat treated. It was verified that there was no loss of flavor during heat treatment up to 80 min.

High Pressure Treatment. In the case of samples with flavor added before high pressure treatment, aliquots (5.194 mL) of the WPI solution (0.5%)/buffer (controls) were transferred into polyallomer Quick-Seal centrifuge tubes (13 mm internal diameter, 51 mm high, Beckman Instruments, Inc., Palo Alto, CA). Using a microliter syringe (Hamilton, Reno, NV), 106 μL of flavor stock solution (50 mg/kg) was added and the tubes were immediately heat sealed. These samples were stored for 40 h at 5 °C for complete equilibration of the free and protein-bound flavor and then transferred to the pressure chamber of the high pressure unit ("Food-Laboratory" food processor, model S-FL-065-200-9-W, Stansted Fluid Power Ltd., Stansted, Essex, UK) and treated at pressures of 250 or 600 MPa for 30 min at 20 °C. An emulsion of 10% vegetable oil in water with surfactant and preservative was used as a pressure-transmitting fluid in the 65 mm × 220 mm cylindrical high pressure chamber. The pressurization and depressurization rates were 3.5 MPa·s^{-1} and 6 MPa·s^{-1}, respectively. The average adiabatic heating during pressurization was ∼2.0 °C/100 MPa, and the cooling rate during depressurization was ∼1.0 °C/100 MPa.

For samples with flavor added after high pressure treatment, 5.3 mL of WPI solution were transferred into the centrifuge tubes, without the

addition of flavor stock solution, and the tubes were heat sealed and high pressure treated as described above.

After the high pressure treatment, samples with added flavor were quantitatively transferred from the centrifuge tubes into 20 mL headspace vials. The vials were immediately sealed with silicone/PTFE septa and magnetic tin crimp caps. For the samples with flavor added after high pressure treatment, 5.194 mL of the pressurized WPI solutions were transferred into 20 mL headspace vials, 106 μL of flavor stock solution was added, the sample vials were sealed, and the samples were equilibrated for 40 h at 5 °C. Samples and controls were then analyzed by headspace SPME and GC-FID.

Automated Headspace SPME-Gas Chromatography. Headspace SPME sampling was performed using a CombiPAL autosampler unit (CTC Analytics AG, Zwingen, Switzerland). After equilibration, the SPME fiber was exposed into the headspace of the sample vial and was subsequently introduced into the gas chromatograph injector port for quantification. The SPME parameters were as follows: 30 μm polydimethylsiloxane (PDMS) fiber (Supelco, Bellefonte, PA), equilibration time 25 min, temperature 30 °C, extraction time 5 min, agitation speed 250 rpm, agitation on time 5 s, agitation off time 2 s, desorption time 5 min. The fiber was conditioned in the gas chromatograph injector port before use at the time and temperature recommended by the manufacturer.

A GC 2010 gas chromatograph coupled with an FID detector (Shimadzu, Kyoto, Japan) was used to quantify the free flavor. The column used was a Supelcowax 10 fused silica capillary column (30 m, inner diameter 0.32 mm, 0.50 μm film thickness) (Supelco, Bellefonte, PA). The carrier gas used was helium (linear velocity 40 $cm \cdot s^{-1}$). The injection port (direct mode) temperature was 250 °C, and the detector temperature was 260 °C. The oven temperature was isothermally held at 120 °C. Data acquisition was achieved using GC Solutions Software (Shimadzu, Kyoto, Japan).

Identification of Unknown Compound. The headspace above the sample solution (10 mL) was extracted for 30 min at 35 °C using a 65 μm PDMS/divinylbenzene (DVB) fiber (Supelco, Bellefonte, PA). A GC 17A gas chromatograph coupled with a QP 5000 mass spectrometer (Shimadzu, Kyoto, Japan) was used to identify the unknown peak. Desorption of the SPME fiber was for 2 min at 250 °C. The column used was an EC 1000 column (30 m, inner diameter 0.25 mm, 0.25 μm film thickness) (Alltech, Deerfield, IL). The carrier gas used was helium (linear velocity 28.8 $cm \cdot s^{-1}$). The injection port (direct mode) temperature was 250 °C, and the detector temperature was 260 °C. The temperature program started at 35 °C, and the temperature was increased to 230 at 5 °C/min and held for 21 min.

Statistical Analysis. All samples were prepared in triplicate, except for the samples with added ascorbic acid and gallic acid, which were prepared in duplicate. The values of % binding of the flavor compounds and WPI were subjected to a *t*-test or analysis of variance. Statistical significance was at $P < 0.05$, and if a significant effect was found, a Tukey's posthoc test was performed. SPSS 14.0 for Windows software (Chicago, IL) was used for statistical evaluations.

RESULTS AND DISCUSSION

Binding of Flavor Compounds to Native WPI. The binding affinity of 2-nonanone, 1-nonanal, and *trans*-2-nonenal to WPI (0.5%) was compared to understand the effect of flavor compound structure on protein−flavor binding (**Figure 1**). At the initial flavor concentration of 1.0 mg/kg, the binding was highest for the unsaturated aldehyde, *trans*-2-nonenal, with 72.3(±0.2)%, followed by the saturated aldehyde, 1-nonanal, with 68.3(±0.1)%, and the saturated methyl ketone, 2-nonanone, with 39.2(±0.5)%.

The presence of the keto group at the end of the C9 chain (1-nonanal) resulted in a significantly higher extent of binding as compared to the keto group in the 2-position (2-nonanone). This can be attributed to the higher hydrophobicity (log P = logarithm of the partition coefficient between *n*-octanol and water) of 1-nonanal as compared to 2-nonanone (**Table 1**). In addition, the aldehyde may also react covalently, e.g., with the

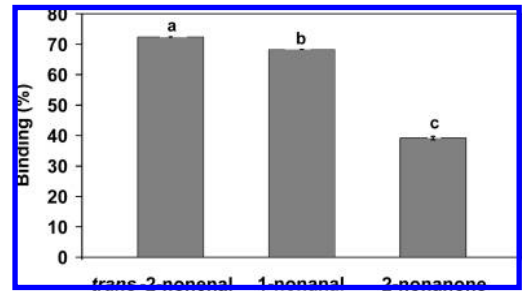


Figure 1. Binding of C9 flavor compounds (1.0 mg/kg) to WPI (0.5%). Different letters indicate significant ($P < 0.05$) differences between samples ($n = 3$).

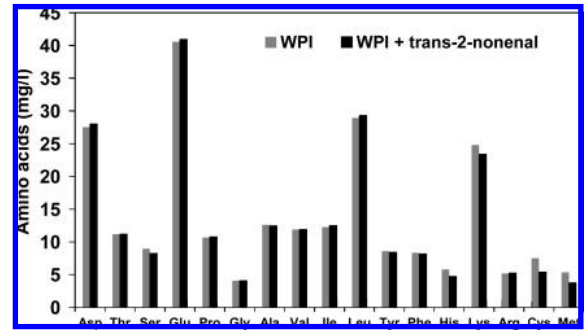


Figure 2. Comparison of amino acids in WPI (0.025%) in the absence and presence of *trans*-2-nonenal (25 mg/kg).

ε-amino group of lysine residues, and therefore show a higher amount of binding than the methyl ketone. For example, hexanal was found to interact with the lysine residues in milk proteins (*5*).

The presence of a double bond further increased the binding affinity of flavor compounds for whey proteins, as seen in the higher binding of *trans*-2-nonenal compared to 1-nonanal (**Figure 1**). *Trans*-2-nonenal is less hydrophobic than 1-nonanal (**Table 1**) but is bound to a higher extent. This suggests possible interactions that are not only hydrophobic in nature, but also could involve the double bond ("Michael addition"). In agreement with this observation, the extent of irreversible binding on soy protein has been shown to be higher for alkenals than for alkanals using a high vacuum transfer method (*21*), and *trans*-2-hexenal has been shown to exhibit covalent binding with milk proteins (*5*). Meynier et al. (*5*) suggested: (a) a reaction of the alkenal double bond with lysine and histidine residues ("Michael addition"), and (b) a reaction of the alkenal aldehyde function with lysine residues (Schiff base formation). Amino acid analysis indicated a reaction of alkenals predominantly with histidine residues but also with lysine and cysteine residues (*5, 22*). Using MS, 4-hydroxy-2-nonenal was found to react with proteins via "Michael addition", whereas only trace amounts of Schiff base were formed (*22*). *Trans*-2-nonenal is therefore very likely to react with WPI to a high extent via "Michael addition".

This hypothesis was confirmed by performing an amino acid analysis of samples containing WPI (0.025%) in the absence and presence of *trans*-2-nonenal (25 mg/kg) (**Figure 2**). Histidine, lysine, cysteine, methionine, and possibly serine reacted with *trans*-2-nonenal, whereas arginine was not modified by the unsaturated flavor compound. Because a number of amino acid residues other than lysine reacted with the flavor compound, the predominant reaction of *trans*-2-nonenal and proteins appears to be an addition reaction rather than a Schiff base formation, which takes place with primary amines, such as lysine.

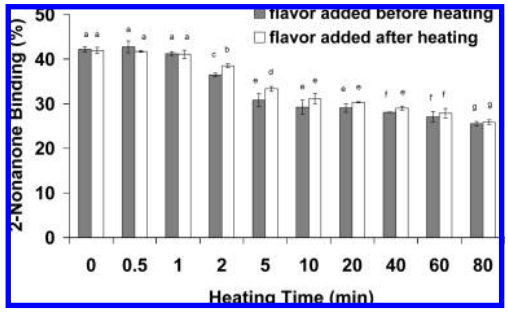


Figure 3. Binding of 2-nonanone (1.0 mg/kg) to WPI (0.5%) with increasing heating time (80 °C) ($n = 3$).

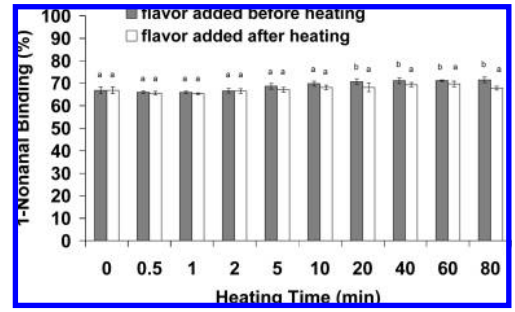


Figure 4. Binding of 1-nonanal (1.0 mg/kg) to WPI (0.5%) with increasing heating time (80 °C) ($n = 3$).

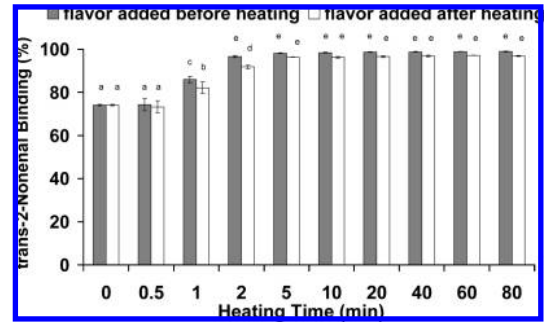


Figure 5. Binding of *trans*-2-nonenal (1.0 mg/kg) to WPI (0.5%) with increasing heating time (80 °C) ($n = 3$).

Effect of Heat Treatment on Flavor Binding to WPI. The binding of 2-nonanone to WPI with increasing heating time at 80 °C is shown in **Figure 3**. In the unheated sample, around 42% of the 2-nonanone initially present was bound to the whey proteins. The binding of 2-nonanone decreased significantly after 2 min of heat treatment for both flavor addition before and after heat treatment. Longer heating times showed a continuous decrease in binding of 2-nonanone, and after 80 min, the binding was only 26%. These results suggest that the flavor perception of 2-nonanone may be increased if whey proteins are denatured. The extent of binding was not influenced by the addition of 2-nonanone before or after heat treatment.

Previously, (*2*) it was demonstrated that β-lg is the protein mainly responsible for flavor binding in WPI and that it possesses one specific binding site for 2-nonanone at flavor compound concentrations up to 0.8 mM (~114 mg/kg). The overall decrease in binding with heat treatment may be explained by the increase in the extent of aggregation of unfolded β-lg molecules, making the flavor binding site inaccessible, or modifying the binding sites on other whey proteins, such as BSA, which was also found to contribute to 2-nonanone binding in solutions of WPI (*2*). Another explanation for the decrease in 2-nonanone binding with heat treatment may be that the exposure of previously buried hydrophobic residues leads to protein−flavor interactions being replaced by protein−protein interactions, resulting in a release of 2-nonanone.

A similar study by O'Neill and Kinsella (*11*), using equilibrium dialysis, also reported a decrease in 2-nonanone binding on β-lg upon heat treatment. In addition, these authors observed a high number of low-affinity binding sites on the denatured protein. The unfolding of β-lg obviously reveals previously buried hydrophobic binding sites that possess a lower affinity for 2-nonanone than the hydrophobic pocket.

Other authors (*16*) suggested that the unfolding of β-lg upon heat denaturation resulted in an increase in the binding of hydrophobic flavor compounds. However, they used benzaldehyde, which may not only interact hydrophobically with proteins but also covalently via its aldehyde function.

Using size-exclusion chromatography, Schokker et al. (*23*) followed the decrease in native β-lg monomers upon heat treatment at 78.5 °C for up to 60 min. Their results can be used to estimate the extent of denaturation during heat treatment in this study because the conditions used were similar (1.7% protein, pH 7.0). After 5 min of heat treatment, approximately 55% of β-lg was still native. Heat treatment for 15 min resulted in less than 20% native β-lg, and after 60 min, less than 10% of the protein remained in the native state. Heat treatment at 80 °C for 80 min is therefore expected to result in no native whey protein left. The amount of binding of 2-nonanone to the denatured WPI is still considerable with around 26% (**Figure 3**) and indicates that large aggregates of unfolded whey proteins are able to bind 2-nonanone.

The effect of heat denaturation on the binding of the corresponding aldehyde, 1-nonanal, is presented in **Figure 4**. When 1-nonanal was added after heat treatment of WPI, the extent of binding between the aldehyde and WPI did not change significantly over the whole range of heating times. However, when the flavor compound was added before the heat treatment, the extent of binding increased significantly after 20 min of heat treatment, as compared to no heating, but remained constant at longer heating times.

These observations suggest a different binding mechanism and different binding sites as compared to 2-nonanone. At neutral pH, aliphatic aldehydes, such as 1-nonanal, can interact both hydrophobically and covalently with proteins (*7*). Because the binding involving hydrophobic interactions appears to decrease upon heat denaturation, as shown for 2-nonanone in **Figure 3** and by O'Neill and Kinsella (*11*), it appears that covalent interactions via the aldehyde function may be increased due to denaturation, resulting in an overall unchanged binding of 1-nonanal with increasing heating time. This is in agreement with the observations of Mills and Solms (*7*), who suggested that an increase in temperature seems to enhance the binding of "reactive" flavor compounds such as aliphatic aldehydes. The observed increase in binding after 20 min in the samples with 1-nonanal added before the heat treatment may be explained with the covalent reaction between the aldehyde function and protein amino groups being favored at the elevated temperature.

The assumption that covalent interactions are facilitated on denatured proteins is supported by the results of the corresponding unsaturated aldehyde, *trans*-2-nonenal, for which a marked increase in binding upon heat treatment was observed (**Figure 5**). After only 1 min of heat treatment, the binding of *trans*-2-nonenal to the whey proteins increased significantly. After 5 min of heat treatment, the binding was close to 100%. This increase in *trans*-2-nonenal binding with denaturation of WPI

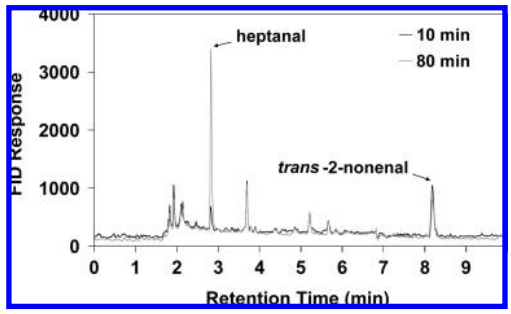


Figure 6. Chromatogram of volatiles after heating WPI (0.5%) and *trans*-2-nonenal (1.0 mg/kg) at 80 °C for 10 and 80 min, respectively.

may be due to covalent reactions of both the aldehyde function and the double bond with certain amino acid residues, such as lysine, histidine, arginine, and cysteine, which may be more readily accessible in the unfolded, aggregated proteins. This is in agreement with results of the amino acid analysis, which revealed that amino acids other than lysine were modified by *trans*-2-nonenal (**Figure 2**).

The increase in binding after 1 and 2 min of heat treatment was significantly more pronounced when *trans*-2-nonenal was added before heat treatment as compared to after heat treatment. This may be explained by the covalent reaction of both the aldehyde function and double bond with amino acid residues being favored at the elevated temperature.

Interestingly, a volatile byproduct was formed in the samples with *trans*-2-nonenal added before heat treatment, eluting at t_r = 2.82 min (**Figure 6**). The additional peak was observed after 10 min of heat treatment. Using MS, this byproduct was tentatively identified as being heptanal, with the typical peaks at *m*/*z* 114 (M^+), 96 (M^+-H_2O), 86 ($C_5H_{10}O$), 81 (M^+-H_2O-CH_3), 70 (C_5H_{10}), 55, and 44 (data not shown). After 80 min of heat treatment, approximately 4% of the bound *trans*-2-nonenal was converted into heptanal.

Heptanal was not formed in samples with *trans*-2-nonenal added after heat treatment, indicating that heat is required for the formation of heptanal. The significantly more pronounced increase in binding when *trans*-2-nonenal was added before heat treatment as compared to after heat treatment (**Figure 5**) may therefore result from the conversion of *trans*-2-nonenal into heptanal. Heptanal was not present in samples heated in the presence of 1-nonanal, showing that the double bond is necessary to form heptanal. Heptanal did not exist in standards containing *trans*-2-nonenal only in buffer (data not shown), indicating that WPI needs to be present to produce heptanal. A reaction of the double bond may be responsible for the formation of heptanal. However, reactions, such as the "Michael addition", form adducts with the proteins and are not known to generate new compounds.

This aspect was not investigated further in this study. Future work should include finding the mechanism of heptanal formation. For example, *N*-terminally blocked amino acids may be used instead of complex proteins to find out if and which amino acid residues in the proteins are involved in the formation of heptanal.

Effect of High Pressure Treatment on Flavor Binding to WPI. The effect of high pressure treatment on the binding of WPI and the three flavor compounds, which were added either before or after high pressure treatment, was investigated. Pressures of 250 and 600 MPa were chosen for this study according to the three stage model proposed by Considine et al. (*24*). In stage I (0.1−150 MPa), the native structure of β-lg is stable; in stage II (200−450 MPa), the native monomer is

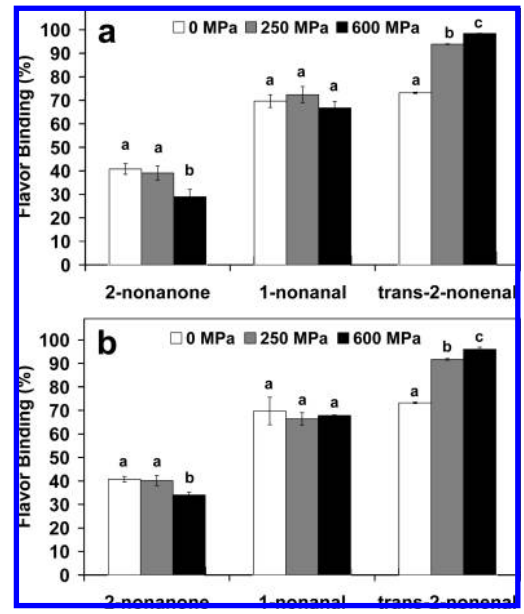


Figure 7. Effect of high pressure treatment (250 and 600 MPa, 30 min) on the binding of 2-nonanone, 1-nonanal, and *trans*-2-nonenal to WPI (0.5%) at pH 7.2. (a) Flavor added before high pressure treatment, (b) flavor added after high pressure treatment. For each individual flavor compound, different letters indicate significant ($P < 0.05$) differences between samples ($n = 3$).

reversibly interchanging with non-native monomers and disulfide bonded dimers; and in stage III (>500 MPa), higher molecular weight aggregates of β-lg are formed.

The samples treated at 600 MPa contained sediment of aggregated proteins, whereas the samples treated at 250 MPa remained clear. High pressure treatment at 250 MPa considerably increased the binding of *trans*-2-nonenal to WPI (**Figure 7**), while the binding of 1-nonanal and 2-nonanone was not affected at this pressure. Pressure treatment at 600 MPa significantly reduced the binding of 2-nonanone to WPI, further increased the binding of *trans*-2-nonenal, but had no effect on the binding of 1-nonanal.

High pressure treatment appears to have the same effect on the protein−flavor interactions as heat treatment. This was expected as both heat and high pressure treatment are known to denature whey proteins.

Under comparable experimental conditions, Yang et al. (*18*) found that high pressure treatment (600 MPa, 32 min, 50 °C) of β-lg (27 μM, pH 7.0) decreased the affinity of the flavor compound capsaicin, while the binding affinity of α-ionone, β-ionone, cinnamaldehyde, and vanillin remained unaffected. Similar results were obtained by Liu et al. (*19*), who observed either no effect or decreased binding of benzaldehyde and methyl ketones (2-heptanone, 2-octanone, 2-nonanone) on high pressure treatment (600 MPa, 10 and 30 min holding time, 50 °C) of whey protein concentrate (WPC), depending on the type and concentration of flavor compound and on the duration of pressurization. For 2-nonanone in particular, the binding slightly decreased after a holding time of 10 min, but after a holding time of 30 min, the binding did not differ from that of an untreated sample. Both studies confirm that the structure of the flavor compound determines its binding affinity on proteins under high pressure.

As observed for heat treatment, there were only very small differences in binding between the samples with flavor added before and after high pressure treatment. In contrast to the heat treated samples, there was no heptanal formation in the samples

with *trans*-2-nonenal added before pressure treatment. To avoid the formation of new flavor compounds, high pressure treatment is therefore the preferred technique of food treatment and preservation as opposed to heat treatment.

This study further elucidated the effects of flavor compound structure and heat and high pressure treatment on the interactions of whey proteins and flavor compounds. The binding between native WPI and the flavor compounds investigated was strong and decreased in the order *trans*-2-nonenal > 1-nonanal > 2-nonanone. The differences in binding can be explained with hydrophobic interactions only in the case of 2-nonanone, whereas the aldehydes, in particular *trans*-2-nonenal, can also interact covalently.

Heat and high pressure treatment affected protein−flavor interactions depending on the structure of the flavor compound. Upon both heat- and pressure-induced denaturation, the binding of 2-nonanone and WPI decreased, while the binding of 1-nonanal remained unchanged, and the affinity for *trans*-2-nonenal increased rapidly. From these observations, it can be concluded that the three flavor compounds investigated are bound on proteins on different binding sites and/or by different binding mechanisms.

The results also suggest that heat- or pressure-induced denaturation reduces hydrophobic interactions of whey proteins with flavor compounds. The decrease in binding of 2-nonanone to WPI upon denaturation can be explained with the destruction of the hydrophobic pocket of β-lg, and with protein−flavor interactions being replaced by protein−protein interactions. The increase in binding of *trans*-2-nonenal may result from mainly covalent binding to amino acid residues, which may be better accessible in the unfolded, aggregated protein molecules. The unchanged binding of 1-nonanal to WPI with heat or high pressure treatment is likely to result from a combination of both a decrease in binding due to the destruction of the hydrophobic pocket and an increase in binding caused by better accessibility of amino acid residues for covalent interactions, resulting in an overall unchanged binding.

For the flavor compounds investigated, there were generally very small differences in binding between the samples with flavor added before and after heat or high pressure treatment. However, at higher flavor concentrations, differences may be observed due to possible stabilizing effects of the flavor compounds on the native conformation of the proteins.

The formation of heptanal upon heating *trans*-2-nonenal in the presence of milk proteins demonstrated that new flavor compounds may be generated during heat treatment under certain conditions, e.g., in the presence of an unsaturated flavor compound. However, the mechanism of heptanal formation is still unclear and requires further investigation.

Heat and high pressure treatment may therefore notably influence the overall flavor profile of protein containing foods. Because the protein concentration used in this study was fairly low, the observed effects are expected to be even more pronounced in real food systems. However, in foods containing even small amounts of fat, the observed effects may not be as pronounced. The impact of denaturation on protein−flavor interactions is of practical significance in food formulations, especially in foods that require pasteurization or UHT treatment, e.g., flavored yoghurts or dairy beverages.

LITERATURE CITED

(1) Hatchwell, L. C. Implications of fat on flavor. In *Flavor−Food Interactions*; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washington, DC, 1996; pp 14−23.

(2) Kühn, J.; Zhu, X. Q.; Considine, T.; Singh, H. Binding of 2-nonanone and milk proteins in aqueous model systems. *J. Agric. Food Chem.* **2007**, *55*, 3599–3604.

(3) Li, Z.; Grün, I. U.; Fernando, L. N. Interaction of vanillin with soy and dairy proteins in aqueous model systems: a thermodynamic study. *J. Food Sci.* **2000**, *65*, 997–1001.

(4) Damodaran, S.; Kinsella, J. E. Flavor protein interactions—binding of carbonyls to bovine serum albumin—thermodynamic and conformational effects. *J. Agric. Food Chem.* **1980**, *28*, 567–571.

(5) Meynier, A.; Rampon, V.; Dalgalarrondo, M.; Genot, C. Hexanal and *t*-2-hexenal form covalent bonds with whey proteins and sodium caseinate in aqueous solution. *Int. Dairy J.* **2004**, *14*, 681–690.

(6) Meynier, A.; Garillon, A.; Lethuaut, L.; Genot, C. Partition of five aroma compounds between air and skim milk, anhydrous milk fat or full-fat cream. *Lait* **2003**, *83*, 223–235.

(7) Mills, O. E.; Solms, J. Interaction of selected flavor compounds with whey proteins. *Lebensm.-Wiss. Technol.* **1984**, *17*, 331–335.

(8) Frapin, D.; Dufour, E.; Haertlé, T. Probing the fatty-acid-binding site of β-lactoglobulins. *J. Protein Chem.* **1993**, *12*, 443–449.

(9) Lübke, M.; Guichard, E.; Le Quéré, J. L. Infrared spectroscopic study of β-lactoglobulin interactions with flavor compounds. In *Flavor Release*; Roberts, D. D., Taylor, A. J., Eds.; American Chemical Society: Washington, DC, 2000; pp 282−292.

(10) Lübke, M.; Guichard, E.; Tromelin, A.; Le Quéré, J. L. Nuclear magnetic resonance spectroscopic study of β-lactoglobulin interactions with two flavor compounds, γ-decalactone and β-ionone. *J. Agric. Food Chem.* **2002**, *50*, 7094–7099.

(11) O'Neill, T. E.; Kinsella, J. E. Effect of heat treatment and modification on conformation and flavor binding by β-lactoglobulin. *J. Food Sci.* **1988**, *53*, 906–909.

(12) Sostmann, K.; Guichard, E. Immobilized β-lactoglobulin on an HPLC-column: a rapid way to determine protein−flavour interactions. *Food Chem.* **1998**, *62*, 509–513.

(13) de la Fuente, M. A.; Singh, H.; Hemar, Y. Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends Food Sci. Technol.* **2002**, *13*, 262–274.

(14) McNeill, V. L.; Schmidt, K. A. Vanillin interaction with milk protein isolates in sweetened drinks. *J. Food Sci.* **1993**, *58*, 1142−1144–1147.

(15) Chobpattana, W.; Jeon, I. J.; Smith, J. S.; Loughin, T. M. Mechanisms of interaction between vanillin and milk proteins in model systems. *J. Food Sci.* **2002**, *67*, 973–977.

(16) Hansen, A. P.; Booker, D. C. Flavor interaction with casein and whey protein. In *Flavor−Food Interactions*; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washington, DC, 1996; pp 75−89.

(17) Iametti, S.; Transidico, P.; Bonomi, F.; Vecchio, G.; Pittia, P.; Rovere, P.; DallAglio, G. Molecular modifications of β-lactoglobulin upon exposure to high pressure. *J. Agric. Food Chem.* **1997**, *45*, 23–29.

(18) Yang, J.; Powers, J. R.; Clark, S.; Dunker, A. K.; Swanson, B. G. Ligand and flavor binding functional properties of β-lactoglobulin in the molten globule state induced by high pressure. *J. Food Sci.* **2003**, *68*, 444–452.

(19) Liu, X.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. *J. Food Sci.* **2005**, *70*, C581−C585.

(20) *AOAC Official Method 994.12, Amino Acids in Feeds*, 16th ed.; AOAC International: Gaithersburg, MD, 1995.

(21) Gremli, H. A. Interaction of flavor compounds with soy protein. *J. Am. Oil Chem. Soc.* **1974**, *51*, A95−A97.

(22) Bruenner, B. A.; Jones, A. D.; German, J. B. Direct characterization of protein adducts of the lipid peroxidation product 4-hydroxy-2-nonenal using electrospray mass spectrometry. *Chem. Res. Toxicol.* **1995**, *8*, 552–559.

(23) Schokker, E. P.; Singh, H.; Pinder, D. N.; Norris, G. E.; Creamer, L. K. Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH. *Int. Dairy J.* **1999**, *9*, 791–800.

(24) Considine, T.; Singh, H.; Patel, H. A.; Creamer, L. K. Influence of binding of sodium dodecyl sulfate, all-*trans*-retinol, and 8-anilino-1-naphthalenesulfonate on the high-pressure-induced unfolding and aggregation of β-lactoglobulin B. *J. Agric. Food Chem.* **2005**, *53*, 8010–8018.

(25) Seuvre, A. M.; Espinosa Diaz, M. A.; Voilley, A. Retention of aroma compounds by β-lactoglobulin in different conditions. *Food Chem.* **2001**, *77*, 421–429.

(26) Rogacheva, S.; Espinosa-Diaz, M. A.; Voilley, A. Transfer of aroma compounds in water−lipid systems: binding tendency of β-lactoglobulin. *J. Agric. Food Chem.* **1999**, *47*, 259–263.

(27) Dannenfelser, R. M.; Yalkowsky, S. H. Database of aqueous solubility for organic nonelectrolytes. *Sci. Total Environ.* **1991**, *109*, 625–628.

(28) Meylan, W. M.; Howard, P. H. Atom fragment contribution method for estimating octanol−water partition coefficients. *J. Pharm. Sci.* **1995**, *84*, 83–92.

Received for review June 12, 2008. Revised manuscript received August 25, 2008. Accepted September 7, 2008.

JF801810B