JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Effect of Enzymatic Deamidation of Soy Protein by Protein−Glutaminase on the Flavor-Binding Properties of the Protein under Aqueous Conditions

Inthawoot Suppavorasatit[†] and Keith R. Cadwallader*[,‡]

[†]Department of Food Technology, Faculty of Science, Chulalongkorn University, Phayatai Road, Wangmai, Pathumwan, Bangkok 10330, Thailand

[‡]Department of Food Science and Human Nutrition, University of Illinois at Urbana−Champaign, 1302 West Pennsylvania Avenue, Urbana, Illinois 61801, United States

ABSTRACT: The effect of the enzymatic deamidation by protein−glutaminase (PG) on flavor-binding properties of soy protein isolate (SPI) under aqueous conditions was evaluated by a modified equilibrium dialysis (ultrafiltration) technique. Binding parameters, such as number of binding sites (n) and binding constants (K), were derived from Klotz plots. The partial deamidation of SPI by PG (43.7% degree of deamidation) decreased overall flavor-binding affinity (nK) at 25 °C for both vanillin and maltol by approximately 9- and 4-fold, respectively. The thermodynamic parameters of binding indicated that the flavor−protein interactions were spontaneous (negative $\Delta G°$) and that the driving force of the interactions shifted from entropy to enthalpy driven as a result of deamidation. Deamidation of soy protein caused a change in the mechanism of binding from hydrophobic interactions or covalent bonding (Schiff base formation) to weaker van der Waals forces or hydrogen bonding.

KEYWORDS: *flavor binding, protein deamidation, soy protein, protein−glutaminase, vanillin, maltol, thermodynamics of binding*

INTRODUCTION

Flavor is a major determinant of consumer acceptance of a food product.[1,2] The availability of a flavor compound for sensory perception is greatly influenced by its interaction with nonvolatile food constituents including fats, proteins, and carbohydrates.[3−5] The binding of flavor compounds to soy protein can lead to a decline in product quality because it can cause flavor fade (loss of flavor or lowering of flavor intensity) or a flavor imbalance due to selective binding of certain flavor compounds over others. This makes it difficult to determine the exact flavoring composition and dose for use in a food formulation.[6]

Flavor−soy protein interactions can be reversible (noncovalent) or irreversible (covalent) depending on the nature of the protein and flavor compounds.[7,8] Whereas most of the interactions are hydrophobic and reversible,[9] irreversible binding can occur for certain flavor compounds, especially carbonyl-containing flavor compounds. These carbonyl groups can form covalent bonds (Schiff bases) with the amine groups of amino acids in proteins.[10,11]

Flavor−protein binding interactions can be altered by protein modification. Deamidation is a protein hydrolysis method that can alter primary, secondary, and tertiary structures of protein by converting amide groups. Deamidation can change the functional properties of a food protein such as solubility, foaming capacity, and emulsification properties[12,13] and also can decrease flavor−protein binding, as demonstrated for a chemically deamidated soy protein isolate.[14] Enzymatic deamidation is generally more desirable than chemical methods because it is substrate specific, can be conducted under mild reaction conditions, and is perceived as natural and safe.[15,16] Protein−glutaminase (PG), first isolated in 2000, catalyzes the deamidation of protein.[17] PG differs from other enzymes with deamidation activity because it does not produce any side reactions, such as cross-linking (TGase) or peptide hydrolysis (protease) and is not limited to the deamidation of glutamine residues in only short peptide chains as with peptidoglutaminase.

In our previous study, we developed a procedure for the deamidation of soy protein isolate (SPI) by using PG, which led to the production of a deamidated protein with modified functional properties.[13] However, that study did not assess the impact of deamidation on the flavor-binding properties of the protein. The present study was aimed at testing the hypothesis that the reduction in the glutamine side chains will reduce the overall flavor-binding affinity of the protein to the carbonyl-containing flavor compounds vanillin and maltol. Therefore, the objective of the present study was to investigate the effect of deamidation on the binding of selected carbonyl-containing flavor compounds to soy protein in an aqueous system using an equilibrium−ultrafiltration method.

MATERIALS AND METHODS

Reagents. Analytical grade (≥98% purity) vanillin (4-hydroxy-3-methoxybenzaldehyde), maltol (3-hydroxy-2-methoxyl-4*H*-pyran-4-one), and ethyl maltol (2-ethyl-3-hydroxy-4*H*-pyran-4-one) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Deuterium-labeled vanillin (vanillin-d_3; 4-hydroxy-3-(methoxy-d_3)-benzaldehyde) was synthesized following the procedure described by Schneider and Rolando.[18]

Received: April 22, 2012
Revised: July 24, 2012
Accepted: July 24, 2012
Published: July 25, 2012

Sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Sigma-Aldrich Co. and used for phosphate buffer preparation. Ethyl ether (99.9% purity, <10.0 ppm BHT) was obtained from Fisher Scientific Inc.

Soy Proteins. SPI (Profam 974) was purchased from Archer Daniels Midland Co. (Decatur, IL, USA) and was vacuum packaged immediately upon receipt. The deamidated SPI (DSPI; 43.7% degree of deamidation and 4.81% degree of hydrolysis) was prepared by PG deamidation for 2 h following the procedure described by Suppavorasatit et al.[13] The DSPI sample was kept in a 125 mL amber glass jar and sealed with a Teflon-lined cap. Both SPI and DSPI were stored at 5 ± 1 °C.

Enzyme. Protein−glutaminase "Amano" 500 (500 U/g) was obtained from Amano Enzyme, Inc. (Elgin, IL, USA).

Preparation of Flavor Compound Solutions. Vanillin (9960 μg/mL) and maltol (10500 μg/mL) stock solutions were prepared in odorless distilled water (prepared by boiling glass-distilled water in an open flask until its volume was reduced by one-third of the original volume). Vanillin-d_3 (1130 μg/mL) and ethyl maltol (1210 μg/mL) solutions were prepared in methanol and used as internal standards. All solutions were kept in 2 mL amber glass vials sealed with Teflon-lined caps and stored at −70 °C.

Isolation of Free Flavor Compounds. A 3 mL aliquot of the reaction mixture was transferred to an Amicon Ultra-4 centrifugal filter tube with 3K molecular weight cutoff (Millipore Corp., Billerica, MA, USA) and centrifuged at 5000*g* for 30 min using a refrigerated centrifuge (Sorvall, Du Pont Co., Wilmington, DE, USA) controlled at the same temperature used for incubation (5, 15, or 25 °C). The permeate was spiked with 20 μL of the vanillin-d_3 (or ethyl maltol) internal standard solution and then thoroughly mixed. One milliliter of the permeate was transferred to a 2 mL glass vial and extracted with 0.5 mL of diethyl ether. The ether fraction was subjected to GC-MS analysis. A flow diagram illustrating the procedure is shown in Figure 1.

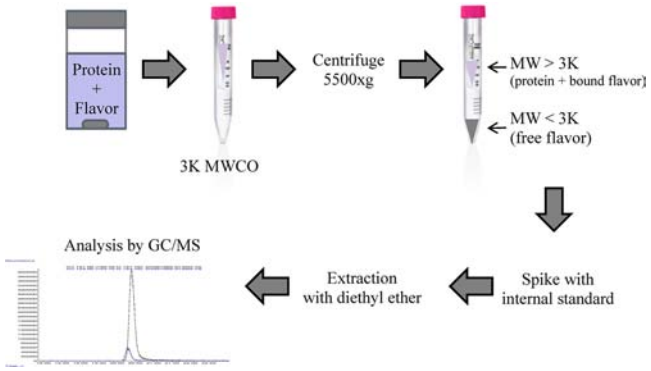


Figure 1. Flow diagram for isolation and quantification of free flavor compounds.

Gas Chromatography−Mass Spectrometry (GC-MS). A series II 5890 GC/5970 mass selective detector (MSD) system (Agilent Technologies, Inc., Palo Alto, CA, USA) was used to quantify vanillin. Two microliters of each sample was injected in the hot splitless mode (250 °C; 30 s valve delay). Separations were performed using an Innowax column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; J&W Scientific, Santa Clara, CA, USA). The oven was programmed from 150 to 220 °C at a rate of 10 °C/min with initial and final holding times of 2 and 20 min, respectively. Helium was used as carrier gas at a constant rate of 1.0 mL/min. The MSD conditions were as follows: transfer line temperature, 250 °C; ionization voltage, 70 eV; mass range (scan mode), 35−400 amu; scan rate, 2 scans/s.

A 6890 GC/5973 MSD system (Agilent Technologies, Inc.) was used to quantify maltol. Two microliters of each sample was injected in the cold splitless mode (initial temperature, −50 °C; initial time, 0.1 min; ramp rate, 12 °C/s; final temperature, 260 °C; valve delay time, 1 min). Separations were performed using a Stabilwax column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Restek, Bellefonte, PA, USA). The oven was programmed from 40 to 225 °C at a rate of 10 °C/min with initial and final hold times of 5 and 20 min, respectively. Helium was used as carrier gas at a constant rate of 1.0 mL/min. MSD conditions were as follows: transfer line temperature, 280 °C; ionization voltage, 70 eV; mass range (scan mode), 35−350 amu; scan rate, 5 scans/s.

Quantification of Free Flavor Compounds. Quantitative analysis was conducted by using MS response factors (f_i) for vanillin and maltol compared against the internal standards (i.s.; vanillin-d_3 or ethyl maltol, respectively). The f_i of each compound is defined as the inverse of the slope of a plot (standard curve) of peak area ratio (flavor compound/i.s.) versus mass ratio (flavor compound/i.s.) for an ascending series of mass ratios. The f_i of vanillin versus vanillin-d_3 [hot splitless injection mode; using mass chromatography peak areas of ion 151 (vanillin) and ion 154 (vanillin-d_3)] was 1.32. For f_i for maltol versus ethyl maltol (cold splitless injection mode; using total ion chromatogram peak areas of maltol and ethyl maltol) was 1.16. The mass of each flavor compound was calculated as follows:

$$\text{mass of flavor compd} = \text{mass of i.s.} \times f_i \times \frac{\text{peak area of flavor compd}}{\text{peak area of i.s.}} \quad (1)$$

Determination of Flavor-Binding Equilibration Times. Prior to use, all glassware was silanized using 10% (v/v) dimethyl dichlorosilane (Sigma-Aldrich Co.) in toluene (Fisher Scientific Inc., Pittsburgh, PA, USA) as described by Tsutsumi and others,[19] then thoroughly rinsed with methanol (Fisher Scientific Inc.), washed, and baked at 190 °C. Protein solutions of SPI or DSPI [3% (w/v)] were prepared in aqueous 0.05 M phosphate buffer (pH 7.0) and then stored at 4 °C overnight to allow for complete hydration. Each protein suspension was placed into a 50 mL test tube equipped with a Teflon-coated magnetic bar. Vanillin (or maltol) was spiked into each suspension to achieve an approximate concentration of 50 μg/mL. The test tubes were sealed with Teflon-lined caps and were incubated with stirring [at speed level 6 using a VWR magnetic stirrer model 310 (VWR International, LLC, Arlington Heights, IL, USA)] at a constant temperature (5, 15, or 25 °C) maintained by using a 1 L low-form jacketed beaker water bath (Chemglass, Inc., Vineland, NJ, USA). At specific time intervals, aliquots of each flavor−protein suspension were withdrawn and the concentration of the free (unbound) flavor compound were determined. Equilibration times were determined from plots of concentration of free flavor compound versus time at constant temperature.

Determination of Binding Properties. The determination of binding properties was performed according to the methods described by Chobpattana et al.[6] and Li et al.[20] with some modifications. Protein solutions of SPI or DSPI [3% (w/v)] were prepared in 0.05 M phosphate buffer (pH 7.0) and then stored at 4 °C overnight to allow for complete hydration of the protein. For binding studies, 5 mL aliquots of each protein suspension were placed into 20 mL glass scintillation vials containing Teflon-coated magnetic stir bars and spiked with vanillin (or maltol) to achieve concentrations of 10, 20, 40, 60, 80, or 100 μg/mL and then sealed with Teflon-lined caps. Solutions of 20 μg/mL vanillin (or maltol) in 0.05 M phosphate buffer (pH 7.0) were used as controls. Each set of vials, consisting of a complete concentration range for each flavor plus the control, were incubated with stirring at three different temperatures (5, 15, or 25 °C) until equilibrium was reached or exceeded (48 h for 5 °C, 36 h for 15 °C, and 24 h for 25 °C), at which point the concentrations of the free (unbound) flavor compounds were determined.

Number of binding sites (n) and binding constants (binding affinity; K) were obtained by generating the double-reciprocal plots (Klotz plots) from the Klotz equation (eq 2). This is one of the most commonly used methods for the analysis of protein−ligand binding data, as previously described:[21,22]

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{Kn[L]} \quad (2)$$

ν is the number of moles of ligand (flavor compound) bound per mole of total protein, and [L] is the concentration of free ligand (free flavor compound). On the basis of eq 2, the double-reciprocal plot of $1/\nu$ versus $1/[L]$ gives a slope equal to $1/Kn$ and a y-intercept equal to $1/n$.

Because the Klotz plot analysis is applicable only to noncovalent interaction between a ligand and a protein, the K determined cannot be treated as a true equilibrium binding constant. Thus, it is probable that the binding might be mainly of a noncovalent nature (i.e., covalent binding is considered to be so low that its contribution to ν is negligible), in which case K can be treated as an equilibrium binding constant.

Determination of Thermodynamic Parameters. Thermodynamic parameters were calculated by using the binding constant (K), derived from the Klotz equation. The Gibb's free energy of binding ($\Delta G°$) for each temperature was calculated from the equation

$$\Delta G° = -RT \ln K \quad (3)$$

where R is the gas constant (1.9859 cal K^{-1} mol^{-1}) and T is the absolute temperature in degrees Kelvin. The enthalpy of binding ($\Delta H°$) was determined from the van't Hoff equation

$$\ln(K_2/K_1) = \frac{\Delta H°/R}{(1/T_2 - 1/T_1)} \quad (4)$$

where K_1 and K_2 are the binding constants at 5 and 25 °C, T_1 and T_2 are the absolute temperatures in degrees Kelvin, and R is the gas constant. The entropy of binding ($\Delta S°$) was determined using the following equation:

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T} \quad (5)$$

Statistical Analysis. Analysis of variance (ANOVA) and least significant difference (LSD) were used to test for differences among treatments ($p < 0.05$) (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

A prerequisite to the use of an equilibrium dialysis technique for flavor-binding studies is knowledge of the minimum incubation time necessary for the system to reach equilibrium under the various experimental conditions to be evaluated. Equilibration times were determined by plotting the concentration of free (unbound) flavor compound as a function of incubation time at 5, 15, and 25 °C for both SPI and DSPI. Typical equilibration curves for binding interactions of SPI or DSPI with vanillin and maltol at 25 °C are shown in Figures 2 and 3, respectively.

The equilibration time was considered to be the minimum time necessary for the free flavor compound to reach a stable (lowest) concentration. Equilibration times for binding of vanillin and maltol to SPI and DSPI at the three experimental temperatures used in this study are given in Table 1.

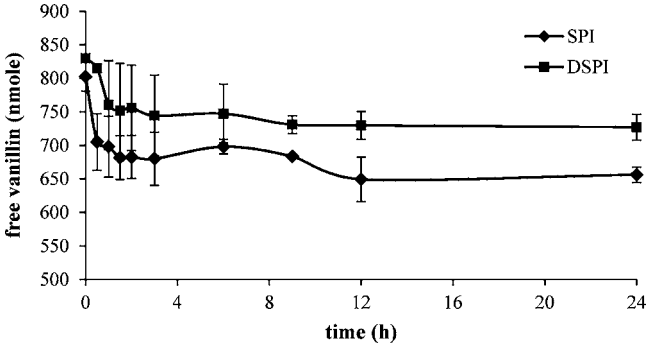


Figure 2. Equilibration curves for binding of vanillin with soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 25 °C.

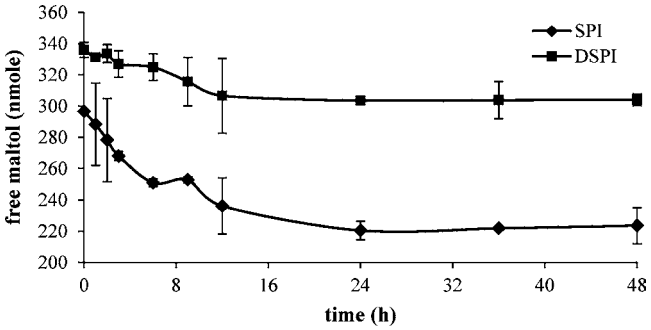


Figure 3. Equilibration curves for binding of maltol with soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 25 °C.

Table 1. Equilibration Time for the Binding of Vanillin and Maltol to Soy Protein Isolate (SPI) and Deamidated Soy Protein Isolate (DSPI) at Different Temperatures

		minimum time to reach equilibrium (h)	
soy protein	T (°C)	vanillin	maltol
SPI	5	48	48
	15	36	36
	25	12	24
DSPI	5	48	48
	15	24	36
	25	9	12

Binding Affinity of Vanillin and Maltol to SPI and DSPI. Panels a, b, and c of Figure 4 show double-reciprocal plots (Klotz plots) for the binding of vanillin to SPI and DSPI at 5, 15, and 25 °C, respectively. The plots are linear, which demonstrates that vanillin binds independently (noncooperative interaction) to both SPI and DSPI over the temperature range studied. These results agree with those of Li and others,[20] who reported noncooperative interaction of vanillin with soy and dairy proteins in an aqueous model system at 4 and 12 °C.

Linear regression equations from the Klotz plots for the binding of vanillin to soy proteins of two replications are presented in Table 2. The coefficients of determination (r^2) of all equations were >0.97, which means that equations can explain >97% of the total variation for the plots.

As mentioned previously, eq 2 was used to determine the number of binding sites (n) and binding constants (binding affinity; K), which were calculated from the y-intercepts ($1/n$) and slopes of the plots ($1/Kn$), respectively. The calculated n and K values for the binding of vanillin to soy proteins are shown in Table 3. The values for n for the interaction of vanillin with SPI at 5, 15, and 25 °C were 13.6, 2.31, and 0.48, respectively. The n values at 5 and 15 °C are in good agreement with those previously reported by Li et al.[20] for the binding of vanillin to SPI at 4 and 12 °C (10.92 and 3.81, respectively). The K values for the binding of vanillin to SPI increased ($p \leq 0.05$) with increasing temperature (Table 3), which agrees with the results of Li and others,[20] who reported that the K values for the binding of vanillin to SPI increased from 468 M^{-1} at 4 °C to 683 M^{-1} at 12 °C.

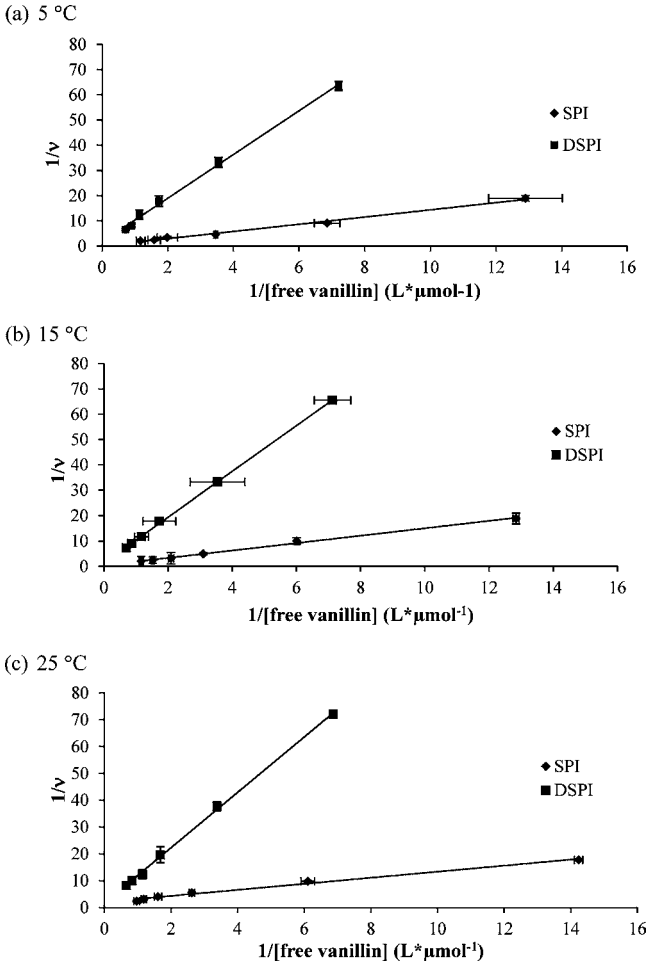


Figure 4. Klotz plots for binding of vanillin to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 5 °C (a), 15 °C (b), and 25 °C (c). Plots represent an average of two complete replications.

However, the magnitudes of the K values calculated in the present study are not close to the values reported by Li et al.[20] This could be due to the fact that the present study was conducted on a different protein source and using a different preparation method (i.e., laboratory prepared in the case of ref 20 vs commercially available SPI in the case of the present study) and by different methodologies, which has been reported to cause systematic differences in K values.[7,13]

The n values for the binding of vanillin to SPI decreased with increasing temperature, whereas the K values increased with increasing temperature (Table 3). However, the n and K values for the binding of vanillin with DSPI were not significantly affected by temperature (Table 3). Values of n were lower for DSPI than SPI at 5 and 15 °C, but did not differ at 25 °C. Furthermore, with respect to vanillin the K values were lower for DSPI than for SPI at 15 and 25 °C, but were higher for DSPI at 5 °C.

The Klotz plots of the binding of maltol to SPI and DSPI at 5, 15, and 25 °C are shown in Figure 5, panels a, b and c, respectively. Linear equations from the Klotz plots for the

Table 3. Binding and Thermodynamic Parameters[a–c] for the Binding of Vanillin to Soy Protein Isolate (SPI) and Deamidated Soy Protein Isolate (DSPI)

parameter	T (°C)	SPI	DSPI
n	5	13.6 ± 0.52 a A	0.63 ± 0.05 ns B
	15	2.31 ± 0.09 b A	0.71 ± 0.06 ns B
	25	0.48 ± 0.06 c NS	0.61 ± 0.05 ns NS
K (×10^4) (M^{-1})	5	5.16 ± 0.46 b B	18.5 ± 0.63 ns A
	15	29.8 ± 1.88 b A	15.7 ± 2.06 ns B
	25	186 ± 25.9 a A	15.9 ± 0.86 ns B
nK (×10^4) (M^{-1})	5	70.4 ± 9.00 ns A	11.5 ± 0.56 a B
	15	68.7 ± 7.11 ns A	11.1 ± 0.50 ab B
	25	88.8 ± 1.33 ns A	9.69 ± 0.21 b B
$\Delta G°$ (kcal mol^{-1})	5	−5.99 ± 0.05 a A	−6.69 ± 0.02 a B
	15	−7.21 ± 0.04 b B	−6.84 ± 0.07 a A
	25	−8.54 ± 0.08 c B	−7.09 ± 0.03 b A
$\Delta H°$ (kcal mol^{-1})	5–25	29.5 ± 1.89 A	−1.22 ± 0.16 B
$\Delta S°$ (cal K^{-1} mol^{-1})	5	106 ± 6.79 ns A	−4.38 ± 0.59 ns B
	15	102 ± 6.55 ns A	−4.22 ± 0.57 ns B
	25	98.9 ± 6.34 ns A	−4.08 ± 0.55 ns B

[a]Within columns, values with the same lower case letters are not significantly different at $p > 0.05$. [b]Within rows, values with the same upper case letters are not significantly different at $p > 0.05$. [c]Average ± standard deviation ($n = 2$).

Table 2. Linear Equations from Klotz Plots for the Binding of Vanillin and Maltol to Soy Protein Isolate (SPI) and Deamidated Soy Protein Isolate (DSPI) Obtained from Two Replications

			replication 1		replication 2	
flavor compd	soy protein	T (°C)	equation	r^2	equation	r^2
vanillin	SPI	5	$y = 1.3026x + 0.0715$	0.9981	$y = 1.5616x + 0.0755$	0.9840
		15	$y = 1.3553x + 0.4217$	0.9973	$y = 1.5693x + 0.4466$	0.9763
		25	$y = 1.1376x + 1.9083$	0.9833	$y = 1.1138x + 2.2768$	0.9937
	DSPI	5	$y = 8.3793x + 1.5106$	0.9940	$y = 8.9729x + 1.6973$	0.9944
		15	$y = 8.7235x + 1.4958$	0.9945	$y = 9.2997x + 1.3234$	0.9965
		25	$y = 10.156x + 1.5559$	0.9986	$y = 10.4793x + 1.7326$	0.9921
maltol	SPI	5	$y = 0.3107x + 0.0942$	0.9984	$y = 0.3000x + 0.0920$	0.9950
		15	$y = 0.3210x + 0.1156$	0.9985	$y = 0.3103x + 0.1229$	0.9960
		25	$y = 0.3073x + 0.2985$	0.9953	$y = 0.3564x + 0.3131$	0.9806
	DSPI	5	$y = 0.4938x + 0.2554$	0.9972	$y = 0.4714x + 0.2433$	0.9985
		15	$y = 0.4665x + 0.2673$	0.9993	$y = 0.4976x + 0.2213$	0.9989
		25	$y = 1.1245x + 0.0335$	0.9997	$y = 1.4234x + 0.0342$	0.9995

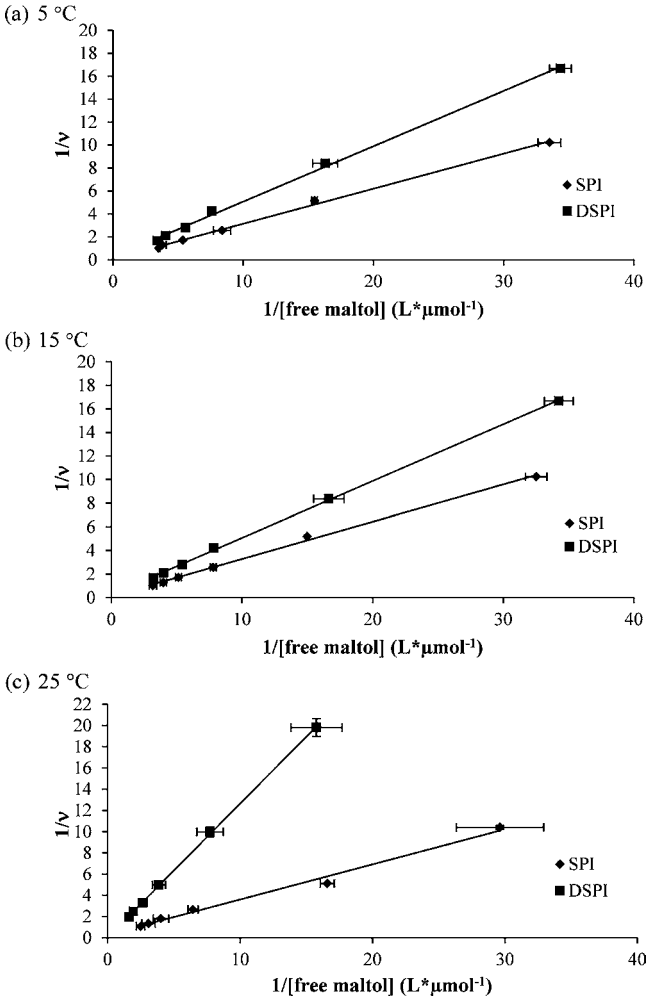


Figure 5. Klotz plots for binding of maltol to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 5 °C (a), 15 °C (b), and 25 °C (c). Plots represent an average of two complete replications.

binding of maltol to soy proteins of two replications are also presented in Table 2. The equations (r^2 = 0.98) explain >98% of the total variation of the plots. These results demonstrate that maltol also was bound noncooperatively to both SPI and DSPI, the same as vanillin as explained above.

The *n* and *K* values for the binding of maltol to soy proteins are shown in Table 4. The *n* values for the binding of maltol to SPI decreased with increasing temperature, whereas the *K* values increased with increasing temperature (Table 4). This is in agreement with the trends in *n* and *K* values observed for the binding interaction of vanillin to SPI (Table 3).

An opposite trend was observed for the binding of maltol to DSPI at 25 °C, where the *n* values increased and the *K* values decreased with increasing temperature. This differs from the results for binding of vanillin to DSPI (Table 3), where temperature had no effect on the values of *n* and *K*.

The decrease in *n* with increasing temperature observed for the binding interaction of vanillin and maltol to SPI can be explained by the increase in protein unfolding as a result of the decrease in temperature.[23] In addition, lower temperatures may cause a rearrangement of protein subunits (dissociation of hydrophobically aggregated protein) due to the weakening of hydrophobic interactions,[24] which could expose a higher number of binding sites at the outer surface of the protein.

Table 4. Binding and Thermodynamic Parameters[a–c] for the Binding of Maltol to Soy Protein Isolate (SPI) and Deamidated Soy Protein Isolate (DSPI)

parameter	*T* (°C)	SPI	DSPI
n	5	10.7 ± 0.18 a A	4.01 ± 0.14 b B
	15	8.38 ± 0.35 b A	4.13 ± 0.55 b B
	25	3.27 ± 0.11 c B	29.5 ± 0.43 a A
K (×10^4) (M^{-1})	5	30.5 ± 0.25 b B	51.7 ± 0.08 a A
	15	37.9 ± 2.47 b NS	50.9 ± 9.07 a NS
	25	92.5 ± 6.57 a A	2.69 ± 0.41 b B
nK (×10^4) (M^{-1})	5	328 ± 8.12 ns A	207 ± 6.80 a B
	15	317 ± 7.60 ns A	208 ± 9.48 a B
	25	303 ± 31.7 ns A	79.6 ± 13.2 b B
$\Delta G°$ (kcal mol^{-1})	5	−6.97 ± 0.01 a A	−7.26 ± 0.00 b B
	15	−7.35 ± 0.04 b NS	−7.51 ± 0.10 b NS
	25	−8.13 ± 0.04 c B	−6.03 ± 0.09 a A
$\Delta H°$ (kcal mol^{-1})	5–25	9.12 ± 0.65 A	−24.3 ± 1.24 B
$\Delta S°$ (cal K^{-1} mol^{-1})	5	32.8 ± 2.34 ns A	−87.6 ± 4.45 ns B
	15	31.7 ± 2.26 ns A	−84.5 ± 4.30 ns B
	25	30.6 ± 2.18 ns A	−81.7 ± 4.16 ns B

[a]Within columns, values with the same lower case letters are not significantly different at $p > 0.05$. [b]Within rows, values with the same upper case letters are not significantly different at $p > 0.05$. [c]Average ± standard deviation (n = 2).

Therefore, more binding sites for vanillin and maltol should be available at 5 °C than at 25 °C.

The above trends, that is, decrease in *n* and increase in *K* with increasing temperature, observed for vanillin and maltol are opposite to what has been reported for some other flavor compounds. Damodaran and Kinsella[24] reported that the *n* value for the binding of 2-nonanone with whole soy protein increased with increasing temperature from 5 to 25 °C, whereas the *K* decreased with increasing temperature. The different behaviors of these flavor compounds could be due to the differences in their hydrophobicities as indicated by the difference in their log *P* values (log *P* of vanillin, 1.19;[25] log *P* of maltol, 1.40;[26] log *P* of 2-nonanone, 2.90[27]), the difference in the functional groups of 2-nonanone (ketone) versus vanillin (aldehyde, phenol, and ether) and maltol (pyranone and hydroxyl), and also the difference in type of soy protein (whole soy protein vs SPI) used in the experiments.

As previously pointed out by Zhou and Cadwallader,[28] use of *n* or *K* alone might not be the best way to represent the overall binding affinity of a flavor compound to a protein. Instead, the value of *nK*, which is derived from the Klotz equation (eq 2), can more accurately measure overall binding affinity.[9,28] The *nK* values (at 5, 15, and 25 °C) demonstrate that the overall binding affinity of vanillin [(68.7−88.8) × 10^4 M^{-1}] or maltol [(303−328) × 10^4 M^{-1}] to SPI was greater than that to DSPI [(9.69−11.5) × 10^4 M^{-1} for vanillin and (79.6−208) × 10^4 M^{-1} for maltol] (Tables 3 and 4). These results indicate that deamidation by PG had a significant effect on the binding of vanillin because the *nK* for DSPI was around 7−9 times lower than for SPI, whereas the *nK* value for the binding of maltol to DSPI was around 1.5−4 times lower than for SPI. It is hypothesized that the overall binding affinities of vanillin and

maltol to DSPI decreased, at least in part, as a result of the loss of reactive amide side groups, in particular, the loss of glutamine resides, on the protein. As a result, DSPI would have less ability to bind to vanillin and maltol via the significant conformational changes in SPI caused by PG. Because the deamidation by PG caused most of the glutamine residues to be replaced by acidic groups, binding of vanillin and maltol to DSPI might also occur via hydrogen-bonding and nonspecific interactions.[14]

Thermodynamics of Binding of Vanillin and Maltol to SPI and DSPI. The thermodynamic parameters for the binding of vanillin and maltol to SPI and DSPI are shown in Tables 3 and 4, respectively. These data indicate a negative free energy of binding ($\Delta G°$) for both flavor compounds to both SPI and DSPI. This means the binding of vanillin and maltol to both proteins was thermodynamically favorable and, thus, was spontaneous. The $\Delta G°$ values indicate that the binding affinities of vanillin to SPI and DSPI were higher at 25 °C than at 5 and 15 °C. Meanwhile, for maltol, the binding affinity was higher for SPI but lower for DSPI at 25 °C. The $\Delta G°$ values determined in the present study for binding of vanillin agree with those of Li and others,[20] who reported $\Delta G°$ values of −3.38 and −3.70 kcal mol^{-1} at 4 and 12 °C, respectively. Taking into consideration the structure of maltol, which contains a ketone group, the $\Delta G°$ value for this compound at 25 °C (−8.13 kcal mol^{-1}) is not much different from the value (−4.1 kcal mol^{-1}) reported by Arora and Damodaran[29] for the binding of 2-nonanone to soy protein in an aqueous system.

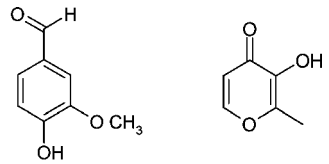
In the present study, the enthalpy of binding ($\Delta H°$) of vanillin to SPI was highly positive (29.5 ± 1.89 kcal mol^{-1}) (Table 3), which indicates that the interaction between vanillin and SPI is not favorable because it is an endothermic reaction. However, the entropy values ($\Delta S°$) were also high (from 98.9 ± 6.3 to 106 ± 6.8 cal K^{-1} mol^{-1}), which resulted in negative $\Delta G°$ values, which indicates that the binding interaction is spontaneous.[20] Similar trends were observed for maltol (Table 4).

Therefore, it can be concluded that the interaction between vanillin or maltol and SPI is an entropy-driven process, which indicates a greater disorder of the system, that is, protein unfolding.[20,30−32] The greater disorder of SPI results in the exposure of new binding sites for the vanillin.[24] Furthermore, all hydrophobic interactions are entropy-driven processes. The increase in entropy comes from positive entropy change of water as the ligand is transferred from the aqueous phase to the protein phase (binding). In addition, if we consider that vanillin has both a carbonyl and an alcohol group in its structure, our results are in agreement with the findings of Aspelund and Wilson.[30] They found that the interactions of SPI with hexanal and 2-hexanone (carbonyl compounds) and 1-hexanol (alcohol) were also entropy-driven.

In contrast to the results for SPI, the values of $\Delta H°$ for the binding of vanillin and maltol to DSPI were negative (−1.22 ± 0.16 and −24.3 ± 1.24 kcal mol^{-1}, respectively). This means that the binding interaction between DSPI and vanillin or maltol was favorable (exothermic). The $\Delta S°$ values for the interaction of both flavor compounds were also negative; however, these were not of high enough magnitude to cause the value of $\Delta G°$ to become positive. Hence, the interactions of vanillin and maltol with DSPI are enthalpy-driven processes.

Previous studies have indicated that the binding of carbonyl-containing flavor compounds to various types of the proteins may be caused by Schiff base formation.[4,10,22] Besides covalent bonding, noncovalent interactions might occur at the same time due to hydrogen bonding, van der Waals forces, and hydrophobic interactions. The $\Delta H°$ and $\Delta S°$ of a reaction can help to identify these binding modes.[33] The values for $\Delta H°$ and $\Delta S°$ for binding of vanillin and maltol to SPI were positive (Table 3), which means that hydrophobic interactions would be involved in the interaction of vanillin and maltol to SPI. With respect to binding interactions with DSPI, the values for $\Delta H°$ and $\Delta S°$ for vanillin and maltol were negative. Therefore, van der Waals force or hydrogen bonding would be involved in the interaction of vanillin and maltol with DSPI.[33,34]

Vanillin and maltol appear to undergo similar binding interactions with SPI and DSPI. This might be because both compounds contain a carbonyl and a hydroxyl functional group (Figure 6). Between the two compounds, maltol showed the greatest overall binding affinity to both SPI and DSPI. This might be due to differences in the orientation of the functional groups on the compounds, thus resulting in different accessibilities to binding sites.



(a) vanillin (b) maltol

Figure 6. Chemical structures of vanillin (a) and maltol (b).

In conclusion, partial enzymatic deamidation of SPI affected the flavor-binding properties of the protein. Vanillin and maltol undergo noncooperative interactions with both SPI and DSPI. The binding of vanillin and maltol onto SPI is entropy-driven, whereas the binding of the two compounds to DSPI is enthalpy-driven. In addition, both vanillin and maltol showed a decrease in binding affinity toward DSPI. The thermodynamic data indicate that vanillin and maltol undergo stronger binding interactions with SPI than with DSPI. It is possible that these differences are due to a shift in the binding mechanisms from predominantly hydrophobic interactions and/or covalent bonding (Schiff base formation) for SPI to mainly van der Waals force or hydrogen bonding in the case of DSPI. These findings may be helpful to soy protein and soy food manufacturers aiming to reduce the flavor fade problem in aqueous protein-containing foods. However, studies on flavor binding by using only an analytical approach cannot demonstrate the actual impact on consumer perception. Thus, further studies using sensory evaluation techniques are still needed.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1-(217)-333-5805. Fax: +1-(217)-333-1875. E-mail: cadwlldr@illinois.edu.

Funding

This project was supported by the USDA National Institute of Food and Agriculture, Hatch Project ILLU-698-366. We thank Amano Enzyme USA Co., Ltd., for providing the PG and the Royal Thai Government Scholarship for the support to I.S.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Liu, K. *Soybeans as Functional Food and Ingredients*; AOCS Press: Champaign, IL, 2005; 331 pp.

(2) Cadwallader, K. R.; Chang, S. K. C. *Chemistry, Texture, And Flavor of Soy*; American Chemical Society: Washington, DC, 2010; 412 pp.

(3) 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.

(4) Guichard, E. Interactions between flavor compounds and food ingredients and their influence on flavor perception. *Food Rev. Int.* **2002**, *18*, 49–70.

(5) Guichard, E. Flavour retention and release from protein solutions. *Biotechnol. Adv.* **2006**, *24*, 226–229.

(6) 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.

(7) Kühn, J.; Considine, T.; Singh, H. Interactions of milk proteins and volatile flavor compounds: implications in the development of protein foods. *J. Food Sci.* **2006**, *71*, R72–R80.

(8) Preininger, M. Interactions of flavor components in foods. In *Ingredient Interactions Effects on Food Quality*, 2nd ed.; Gaonkar, A. G., McPherson, A., Eds.; CRC Press: Boca Raton, FL, 2006; pp 477–542.

(9) Kühn, J.; Considine, T.; Singh, H. Binding of flavor compounds and whey protein isolate as affected by heat and high pressure treatments. *J. Agric. Food Chem.* **2008**, *56*, 10218–10224.

(10) Hansen, A. P.; Heinis, J. J. Decrease of vanillin flavor perception in the presence of casein and whey proteins. *J. Dairy Sci.* **1991**, *74*, 2936–2940.

(11) Damodaran, S. Amino acids, peptides, and proteins. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; CRC Press: Boca Raton, FL, 1996; pp 321–429.

(12) Hamada, J. S. Deamidation of food proteins to improve functionality. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 283–292.

(13) Suppavorasatit, I.; de Mejia, E.; Cadwallader, K. R. Optimization of the enzymatic deamidation of soy protein by protein–glutaminase and its effects on the functional properties of the protein. *J. Agric. Food Chem.* **2011**, *59*, 11621–11628.

(14) Lozano, P. R. *Investigation of Single and Competitive Binding of Volatile Flavor Compounds by Soy Proteins in Low-Moisture Soy Systems*. Ph.D. thesis, Department of Food Science and Human Nutrition, University of Illinois at Urbana–Champaign, 2009; p 131.

(15) Hamada, J. S. Peptidoglutaminase deamidation of proteins and protein hydrolysates for improved food use. *J. Off. Anal. Chem. Soc.* **1991**, *68*, 459–462.

(16) Shih, F. F. Deamidation and phosphorylation for food protein modification. In *Surface Activity of Proteins. Chemical and Physicochemical Modifications*; Magdassi, S., Ed.; Dekker: New York, 1996; pp 91–113.

(17) Yamaguchi, S.; Yokoe, M. A novel protein-deamidating enzyme from *Chryseobacterium proteolyticum* sp. nov., a newly isolated bacterium from soil. *Appl. Environ. Microbiol.* **2000**, *66*, 3337–3343.

(18) Schneider, S.; Rolando, C. One step synthesis of vanillin d_3 (4-hydroxy-3-(methoxy d_3)-benzaldehyde). *J. Labelled Compd. Radiopharm.* **1992**, *31*, 489–492.

(19) Tsutsumi, H.; Nishikawa, M.; Katagi, M.; Tsuchihashi, H. Adsorption and stability of suxamethonium and its major hydrolysis protduct succinylmonocholine using liquid chromatography-electrospray ionization mass spectrometry. *J. Health Sci.* **2003**, *49* (4), 285–291.

(20) 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.

(21) Klotz, I. M.; Walker, F. M.; Pivan, R. B. The binding of organic ions by proteins. *J. Am. Chem. Soc.* **1946**, *68*, 1486–1490.

(22) Suppavorasatit, I.; Cadwallader, K. R. Flavor–soy protein interactions. In *Chemistry, Texture, And Flavor of Soy*; Cadwallader, K. R., Chang, S. K. C., Eds.; American Chemical Society: Washington, DC, 2010; pp 339–359.

(23) Pastore, A.; Martin, S. R.; Politou, A.; Kondapali, K. C.; Stemmler, T.; Temussi, P. A. Unbiased cold denaturation: low- and high-temperature unfolding of yeast frataxin under physiological conditions. *J. Am. Chem. Soc.* **2007**, *129*, 5374–5375.

(24) Damodaran, S.; Kinsella, J. E. Interaction of carbonyls with soy protein: thermodynamic effects. *J. Agric. Food Chem.* **1981**, *29*, 1249–1253.

(25) Kikuchi, S.; Kobayashi, T.; Adachi, S. Dependences of the distribution coefficients of hydrophobic solutes on porous methyl methacrylate resin on the temperature and methanol content of the eluent. *Food Sci. Technol. Res.* **2008**, *14* (2), 144–147.

(26) Enyedy, É. A.; Hollender, D.; Kiss, T. Lipophilicity of kinetically labile metal complexes through the example of antidiabetic Zn(II) and VO(IV) compounds. *J. Pharm. Biomed.* **2011**, *54*, 1073–1081.

(27) Seuvre, A. M.; Diaz, M. A. E.; Voilley, A. Transfer of aroma compounds through the lipidic-aqueous interface in a complex system. *J. Agric. Food Chem.* **2002**, *50*, 1106–1110.

(28) Zhou, Q.; Cadwallader, K. R. Effect of flavor compound chemical structure and environmental relative humidity on the binding of volatile flavor compounds to dehydrated soy protein isolates. *J. Agric. Food Chem.* **2006**, *54*, 1838–1843.

(29) Arora, A.; Damodaran, S. Competitive binding of off-flavor compounds with soy protein and β-cyclodextrin in a ternary system: a model study. *J. Am. Oil Chem. Soc.* **2010**, *87*, 673–679.

(30) Aspelund, T. G.; Wilson, L. Adsorption of off-flavors compounds onto soy protein: a thermodynamic study. *J. Agric. Food Chem.* **1983**, *31*, 539–545.

(31) O'Keefe, S. F.; Resurreccion, A. P.; Wilson, L. A.; Murphy, P. A. Temperature effect on binding of volatile flavor compounds to soy protein in aqueous model systems. *J. Food Sci.* **1991**, *56*, 802–806.

(32) Price, N. C.; Dwek, R. A.; Ratcliffe, R. G.; Wormald, M. R. Basic thermodynamics. In *Principles and Problems in Physical Chemistry for Biochemists*, 3rd ed.; Oxford University Press: New York, 2001; pp 11–33.

(33) Wang, C.; Li, Y. Study on the binding of propiconazole to protein by molecular modeling and a multispectroscopic method. *J. Agric. Food Chem.* **2011**, *59*, 8507–8512.

(34) Ross, P. D.; Rekharsky, M. V. Thermodynamics of hydrogen bond and hydrophobic interactions in cyclodextrin complexes. *Biophys. J.* **1996**, *71*, 2144–2154.