Flavor−Protein Binding: Disulfide Interchange Reactions between Ovalbumin and Volatile Disulfides

Rachel L. Adams,[†] Donald S. Mottram,*[,†] Jane K. Parker,[†] and Helen M. Brown[‡]

School of Food Biosciences, The University of Reading, Whiteknights, Reading RG6 6AP, United Kingdom and Campden and Chorleywood Food Research Association, Chipping Campden, Gloucestershire, GL55 6LD, United Kingdom

The irreversible binding of selected sulfur-containing flavor compounds to proteins was investigated in aqueous solutions containing ovalbumin and a mixture of disulfides (diethyl, dipropyl, dibutyl, diallyl, and 2-furfuryl methyl) using solid-phase micro-extraction (SPME). In systems which had not been heated, the recovery of disulfides from the headspace above the protein at the native pH (6.7) was similar to that from an aqueous blank. However, significant losses were observed when the pH of the solution was increased to 8.0. When the protein was denatured by heating, much greater losses were observed and some free thiols were produced. In similar heat-denatured systems at pH 2.0, no losses of disulfides were observed. Disulfides containing allyl or furfuryl groups were more reactive than saturated alkyl disulfides. Interchange reactions between protein sulfhydryl groups and the disulfides are believed to be responsible for the loss of the disulfides.

Keywords: *Disulfides; sulfhydryl interchange; proteins; flavor binding; SPME*

INTRODUCTION

Binding of flavor compounds to proteins has been the subject of a number of studies and reviews (*1*−*3*). The role of flavor binding in off-flavor development in certain foods, particularly those containing soy proteins, has also been examined (*4*−*6*). However, an area which has received relatively little attention is the binding of sulfur-containing flavor compounds with proteins, particularly covalent binding, which is believed to occur between disulfides and protein.

Sulfur-containing compounds are extremely important in cooked foods for roast, savory, meaty, and coffee-like aromas and flavors (*7*). They have low odor threshold values and, therefore, relatively small quantities in a food product can have a major influence on the aroma. Irreversible binding of such compounds in food matrixes would result in a reduction in headspace concentration, and thus alter the flavor perception of the food. This has implications for consumer acceptance of food products where these compounds are used as flavorings or where they occur naturally.

Recently, it was shown that when two disulfides, bis-(2-methyl-3-furyl) disulfide and bis(2-furfuryl) disulfide, were added to a meat system (minced beef) and to ovalbumin, significant proportions of the disulfides were broken down to corresponding thiols (2-methyl-3-furanthiol and 2-furanmethanethiol, respectively) and some were lost completely (*8, 9*). In addition, small amounts of mixed disulfides (2-methyl-3-furyl methyl disulfide and 2-furfuryl methyl disulfide) were formed in the meat system. An aqueous blank, which was used as a control, showed no breakdown of the disulfides. Further experiments using casein, which is less rich in cysteine units, showed very little conversion of disulfides to thiols. When maltodextrin was used as the substrate, no changes in disulfides were observed compared with the aqueous blank. This research indicated that a covalent interaction between the protein and the disulfides, or the corresponding thiol, had occurred, probably through disulfide interchange reactions.

Solid-phase micro-extraction (SPME) is a solventless method of extracting volatiles from gaseous, liquid, or solid phases (*10*−*12*), and it has been widely used in the analysis of flavor profiles of many foods. This paper reports on the application of SPME for the analysis of volatile disulfides in aqueous systems and its use for investigations into the effect of pH on the binding of disulfides to both native and heat-denatured ovalbumin systems.

MATERIALS AND METHODS

Materials. Disulfides (diethyl, dipropyl, dibutyl, and 2-furfuryl methyl) and propyl propanoate were purchased from Aldrich Chemical Co. (Dorset, UK). Diallyl disulfide was purchased from Fluka (Dorset, UK). Ovalbumin (grade III, minimum 90%) and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Sigma Chemical Company (Dorset, UK). All solutions were prepared using HPLC-grade water (Rathburn Chemicals Ltd, Walkerburn, UK). The SPME fiber and holder were purchased from Supelco Chromatography Products (Dorset, UK).

Preparation of Solutions. A stock solution containing 1000 mg/L of each disulfide (diethyl, dipropyl, dibutyl, diallyl, and 2-furfuryl methyl disulfide) was made up in ethanol. Further dilutions in HPLC-grade water were made to give a solution containing 10 mg/L of each disulfide. A separate solution containing a reference standard (propyl propanoate) was made up in a similar way. Aliquots (1 g) of these solutions were weighed out accurately (± 0.05 g), added to a 1% aqueous solution of ovalbumin, and made up to 100 mL to give a concentration of 100 μg/L of each compound. A control system in water was also prepared. The pH of the native ovalbumin solutions was 6.7. Some systems were also examined at pH 8.0 and pH 2.0 in which the pH was adjusted using 0.1M NaOH or 0.1 M HCl, respectively. The effect of the denaturation by heating was examined by heating solutions in closed

* To whom correspondence should be addressed (fax +44 118 931 0080; e-mail D. S.Mottram@reading.ac.uk).

[†] The University of Reading.

[‡] Campden & Chorleywood Food Research Association.

10.1021/jf0100797 CCC: $20.00
Published on Web 08/17/2001

Table 1. Disulfides Extracted from the Headspace above Aqueous Systems with and without Unheated Protein Using SPME

	water		pH 6.7[c]		pH 8.0[d]	
compound	mean[a]	CV[b]	mean	CV	mean	CV
propyl propanoate	1349	10	1196	2	1271	4
diethyl disulfide	1428	2	1052	4	836	2
diallyl disulfide	1073	7	907	1	734	1
dipropyl disulfide	1631	4	1471	3	1188	3
2-furfuryl methyl disulfide	337	8	269	9	192	2
dibutyl disulfide	1777	2	1592	4	1487	4

[a] Mean peak areas of three determinations (arbitrary units). [b] % Coefficient of variation. [c] Native protein at pH 6.7. [d] pH of protein solution adjusted to 8.0.

100-mL glass bottles in a boiling waterbath for 10 min. The solutions attained a temperature of 90 °C, and the pH of the systems did not change on heating. The disulfide mixture was added to the protein solution, either before heating (BH) or after heating (AH).

Solid-Phase Micro-Extraction (SPME) of Ovalbumin Systems. The headspace volatiles were analyzed by SPME using a carboxen-poly(dimethylsiloxane) fiber with a phase thickness of 75 μm. Aliquots (20 g) were weighed out accurately ($\pm$ 0.1 g) into 40-mL screw-top SPME vials and were left for 30 min at room temperature. The fiber was then exposed to the sample headspace for 30 min ($\pm$ 0.2 min) while the vial was maintained at 37 °C. After headspace collection, the fiber was desorbed in the injection port (at 250 °C) for 3 min, then purged for a further 10 min before the next analysis. Analyses were carried out in quadruplicate.

GC Analysis. A Hewlett-Packard 5890 gas chromatograph with a flame ionization detector was equipped with a 60 m $\times$ 0.25 mm i.d. CP Sil 8 CB MS column (Chrompack Ltd). The volatiles were thermally desorbed from the SPME fiber at 250 °C in the split/splitless injection port and cryofocused directly onto the front of the GC column, while the oven was held at 0 °C for 3 min. The oven temperature was raised to 40 °C over 1 min and held for a further 2 min before being raised to 180 °C at a rate of 4 °C/min. The helium carrier-gas flow rate was 1 mL/min.

GC-MS Analysis. A Hewlett-Packard 5890/5972 gas chromatograph−mass spectrometer was used, with the same column, fiber desorption, and operating conditions as those used in the GC analysis. Mass spectra were recorded in the electron impact mode at an ionization voltage of 70 eV and a source temperature of 200 °C. A scan range of 29−400 amu with a scan time of 0.69 s was used. The compounds were identified by comparison of retention times and mass spectra with those of authentic compounds.

Determination of Sulfhydryl Groups in Proteins. Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) was made up to 3 mM by dissolving 0.12 g in 100 mL of phosphate buffer (0.1 M, pH 7.3). Ovalbumin solutions (1%) were prepared by dissolving 0.5 g in 50 mL of HPLC-grade water and adjusting the pH to 8.0 with 0.1 M NaOH. Some of the protein solutions were subjected to heat (100 °C for 10 min), whereas others were used in their native state at room temperature. The concentration of protein in the solutions was estimated by measuring the absorbance at 280 and 320 nm on a CECIL 1000 series (CE 1021) single-beam spectrophotometer (ϵ ovalbumin = 27306 M^{-1} cm^{-1} at 280 nm; *19*). The absorbance at 320 nm was used to correct for any turbidity in the protein solution. Ellman's reagent (0.2 mL) was added to 4 mL of the protein solution, vortexed for 1 min, and left for 10 min for the yellow color to develop. The absorbance was then measured at 412 nm and the concentration of SH-groups was estimated (ϵ nitromercaptobenzoic acid = 13600 M^{-1} cm^{-1}).

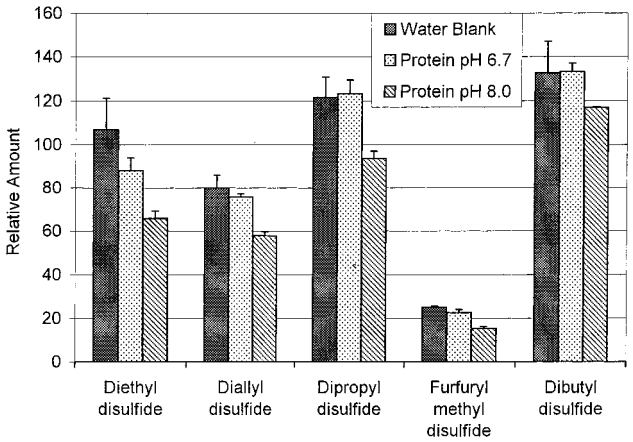

Figure 1. Relative amounts of disulfides in the headspace above aqueous systems with and without native unheated ovalbumin using SPME, measured relative to propyl propanoate (= 100).

RESULTS AND DISCUSSION

Measurement of the volatile aroma compounds in the headspace above the aqueous ovalbumin solutions using SPME showed good reproducibility between replicate analyses (Tables 1 and 2). Half the compounds analyzed showed percent coefficients of variation (CV) of less than 5%, and, with one exception, the only compounds with % CV greater than 10% were compounds present at low concentrations in the headspace of the heated ovalbumin systems. The headspace concentration of aroma compounds above an aqueous solution containing protein will depend on a number of factors relating to the protein, such as hydrophobic bonding or solution viscosity. Propyl propanoate was used as an internal standard with the assumption that it would be subjected to physical interactions similar to those of the disulfides but would not bind covalently. This provided compensation for changes in the headspace concentration due to factors other than covalent interaction between protein and the disulfides.

The results reported here have shown that it is possible to attain good levels of reproducibility using SPME for the extraction of sulfur-containing compounds. This is in agreement with other work using SPME for the analysis of sulfur containing compounds (*13*, *14*).

Figure 1 shows relative changes in disulfides in unheated protein systems compared to those of a water

Table 2. Disulfides Extracted from the Headspace above Aqueous Systems with and without Heated Protein Using SPME

	water		pH 6.7 (BH)[a]		pH 6.7 (AH)[b]		pH 8.0 (BH)		pH 8.0 (AH)	
compound	mean[c]	CV[d]	mean	CV	mean	CV	mean	CV	mean	CV
propyl propanoate	1205	7	1202	3	1356	1	1311	2	1378	3
diethyl disulfide	1444	7	910	5	1338	3	629	5	1196	7
diallyl disulfide	1080	15	121	18	597	14	30	23	353	29
dipropyl disulfide	1721	8	521	10	1118	5	252	4	791	10
2-furfuryl methyl disulfide	275	7	7	35	72	27	ND	ND	31	54
dibutyl disulfide	1800	3	262	11	676	2	128	6	487	7

[a] Disulfides added before heating. [b] Disulfides added after heating. [c] Mean peak areas of four determinations; ND, not detected. [d] % Coefficient of variation.

$$PSH \rightleftharpoons PS^- + H^+$$

$$PS^- + RS\text{–}SR \rightleftharpoons PS\text{–}SR + RS^-$$

$$RS^- + PS\text{–}SP \rightleftharpoons RS\text{–}SP + PS^-$$

$$RS^- + H^+ \rightleftharpoons RSH$$

Figure 2. Simple ionic disulfide interchange reaction in aqueous solution (adapted from Whitesides et al. (*15*)). P = protein chain.

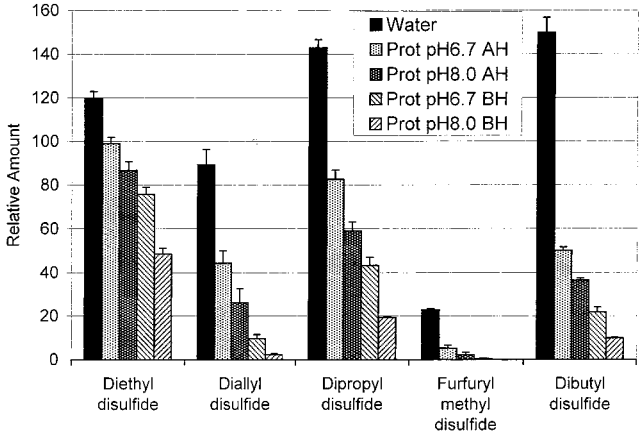

Figure 3. Relative amounts of disulfides in the headspace above aqueous systems of heat-denatured ovalbumin compared to a water blank using SPME, measured relative to propyl propanoate (= 100). BH = Disulfides added before heating; AH = disulfides added after heating.

blank. The pH of the native ovalbumin solution was 6.7 and, at this pH, there was very little difference in recovery of disulfides between the ovalbumin system and the aqueous blank. However, when the pH was adjusted to 8, using NaOH, greater losses were observed. This suggests that a pH-dependent reaction occurred between the disulfides and the protein systems. A mechanism for the interaction between protein sulfhydryl groups and disulfides was proposed by Whitesides et al. (*15*), which requires the initial ionization of the sulfhydryl group in the protein to give a thiolate anion (Figure 2). At pH 8, which is close to the pK_a of cysteine SH in a protein environment (8.80) (*16*), more of the sulfhydryl groups would be present as the more reactive thiolate anion.

The effect of thermal denaturation of the ovalbumin on the recovery of disulfides from the aqueous systems is shown in Figure 3. The losses of disulfides were greater with the heat-denatured protein than with the native ovalbumin. This indicates that more free sulfhydryl groups were present in the denatured protein than in the native state. Two different heated systems were examined: systems where the disulfides were added before heating (BH) and those where they were added after heating (AH). These represent different ways in which flavorings may be incorporated into food products. More disulfides were lost from the former, which could be due to greater reaction rate at the higher temperature, but also the availability of the sulfhydryl groups would be maximized as the protein unfolded during the denaturation. The same effect of pH, as seen in the unheated protein, was observed in the heated systems. The systems at pH 6.7, both for compounds added before and after heating, lost fewer disulfides than for the similar systems at pH 8.0.

Confirmation that an ionic mechanism, as proposed in Figure 2, was likely to be involved in the sulfhydryl–disulfide interchange reactions was provided by examining the interaction of disulfides with ovalbumin at pH 2 when no significant dissociation of the sulfhydryl group would occur. Figure 4 shows that under these acidic conditions there was no loss of disulfides from either the unheated or heated ovalbumin systems.

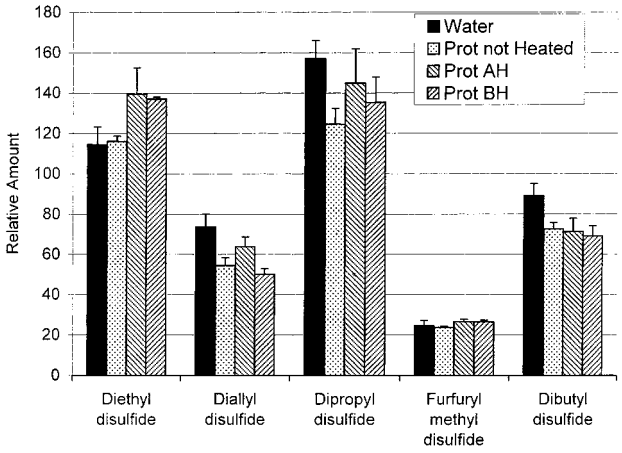

Figure 4. Relative amounts of disulfides in the headspace above aqueous systems containing ovalbumin at pH 2.0, measured relative to propyl propanoate (= 100). BH = Disulfides added before heating; AH = disulfides added after heating.

Table 3. Disulfides and Thiols Extracted from the Headspace above Water and Heat-Denatured Protein Systems Analyzed by GC–MS

	water		protein[a]	
compound	mean[b]	CV[c]	mean	CV
allyl mercaptan	ND	ND	34	5
1-propanethiol	ND	ND	31	3
1-butanethiol	ND	ND	60	2
2-furanmethanethiol	ND	ND	9	3
diethyl disulfide	196	5	93	7
diallyl disulfide	140	3	8	1
dipropyl disulfide	269	5	50	9
2-furfuryl methyl disulfide	55	1	ND	ND
dibutyl disulfide	295	8	40	7
allyl sulfide[d]	25	1	20	3

[a] Protein pH 8.0 disulfides added before heating. [b] Amount relative to internal standard of propyl propanoate; ND, not detected. [c] % Coefficient of variation. [d] The allyl sulfide was an impurity in the diallyl disulfide.

Two systems were also analyzed by GC–MS: the blank water system and the protein at pH 8.0 with the disulfides added before heating (i.e., the system showing the greatest loss of disulfides). The results shown in Table 3 clearly demonstrate that some of the disulfides in the protein system were broken down to their corresponding thiols. Ethanethiol may also have been produced, however, it eluted too early in the chromatogram for it to be identified under the GC conditions used. The diallyl disulfide contained approximately 20% diallyl sulfide present as an impurity. It was interesting to note that the diallyl sulfide did not react, even under the most severe conditions, thus further supporting the hypothesis that the disulfides are lost through an interchange reaction in which diallyl sulfide cannot readily participate.

Sulfhydryl–disulfide interchange reactions are well-known in protein chemistry (*17*) and, in flour, sulfhydryl–disulfide interchanges between glutathione and the flour proteins are important in relation to dough rheology and bread-making (*18*). The mechanism shown in Figure 2 summarizes the pathway by which sulfhy-

dryl groups could initiate the reaction between protein and disulfides, resulting in a net loss of flavor disulfides. As discussed above, the initial step is the dissociation of the sulfhydryl group on the protein, which is followed by the nucleophilic attack of the resulting thiolate anion on a sulfur atom of the flavor disulfide group. Subsequently, attack of the newly generated thiolate anion on the sulfur atoms of the disulfide bonds in the protein occurs. Protonation of the thiolate ion, produced from the flavor disulfide, results in the formation of a free thiol. These free thiols are more likely to be formed when the availability of the protein disulfides is low. The diallyl and the furfuryl methyl disulfides showed greater reactivity than the alkyl disulfides toward the ovalbumin, presumably because the unsaturation in these molecules made the disulfide group more susceptible to nucleophilic attack.

Because it is the dissociation of the sulfhydryl group of the protein that initiates the interchange mechanism between the proteins and the flavor compounds, the number of available sulfhydryl groups present and the extent of their dissociation in the different ovalbumin systems could explain the different degrees of interchange observed in the systems. Available sulfhydryl groups in the systems were estimated using Ellman's method (*19*, *20*). This is based on the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with free sulfhydryl groups in which the reagent is reduced to form 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color and can be used quantitatively as an indirect measure of SH groups.

Although the ovalbumin molecule has four free sulfhydryl groups and one disulfide bond per molecule, by using Ellman's method the native ovalbumin was found to have only 0.01 available sulfhydryl groups per molecule. However, this can be explained by the fact that, in the native state, the sulfhydryl groups of ovalbumin are buried within the tertiary structure of the protein and are unavailable to react with Ellman's reagent (*21*). However, in the heat-denatured state, Ellman's reagent showed that 1.24 sulfhydryl groups per molecule were exposed, which represents a 200-fold excess of protein SH groups compared with the added disulfides. This accounts for the fact that there was a greater loss of disulfides in the denatured state and supports the hypothesis that the free sulfhydryl groups initiate the disulfide exchange reaction and the binding of the flavor disulfides to the protein.

CONCLUSIONS

Interchange reactions between disulfide aroma compounds and ovalbumin resulted in a loss of disulfides and the formation of some free thiols. The extent of the reaction depended on the group attached to the disulfide bond of the aroma compound, on the denaturation state of the protein, and on the degree of dissociation of the protein sulfhydryl groups. Only very small losses of the disulfides were observed in the systems containing native unheated protein, but the losses increased when the protein was heated. The loss of disulfides appeared to be directly related to the availability of the sulfhydryl groups in the denatured protein.

LITERATURE CITED

(1) Solms, J. Interactions of nonvolatile and volatile substances in foods. In *Interactions of Food Components*; Birch, G. G., Lindley, M. G., Eds.; Elsevier Applied Science: London, 1985; pp 189−210.

(2) Bakker, J. Flavor interactions with the food matrix and their effects on the perception. In *Ingredient Interactions − Effects on Food Quality*; Gaonkar, A. G., Ed.; Marcel Dekker: New York, 1995; pp 411−439.

(3) Lubbers, S.; Landy, P.; Voilley, A. Retention and release of aroma compounds in foods containing proteins. *Food Technol.* **1998**, *52*, 68−74, 208−214.

(4) Kalbrener, J. E.; Eldridge, A. C.; Moser, H.; Wolf, W. J. Sensory evaluation of commercial soy flours, concentrates and isolates. *J. Cereal Chem.* **1971**, *48*, 595−600.

(5) Kinsella, J. E. Texturized proteins: fabrication, flavoring and nutrition. *Crit. Rev. Food Sci. Nutr.* **1978**, *10*, 147−207.

(6) Kinsella, J. E.; Damodaran, S. Flavour problems in soy proteins: origin, nature, control and binding phenomena. In *The Analysis and Control of Less Desirable Flavors in Food and Beverages*; Charamboulas, G., Ed.; Academic Press: New York, 1980; pp 95−131.

(7) Mottram, D. S. Flavour formation in meat and meat products: a review. *Food Chem.* **1998**, *62*, 415−424.

(8) Mottram, D. S.; Szauman-Szumski, C.; Dodson, A. Interaction of thiol and disulfide flavor compounds with food components. *J. Agric. Food Chem.* **1996**, *44*, 2349−2351.

(9) Mottram, D. S.; Nobrega, I. C. C.; Dodson, A. T. Extraction of thiol and disulfide aroma compounds from food systems. In *Flavor Analysis: Developments in Isolation and Determination*; Mussinan, C. J., Morello, M. J., Eds.; American Chemical Society: Washington, DC, 1998; pp 78−84.

(10) Arthur, C. L.; Pawliszyn, J. Solid-phase micro-extraction with thermal desorption using fused silica optical fibers. *Anal. Chem.* **1990**, *62*, 2145−2148.

(11) Zhang, Z.; Pawliszyn, J. Headspace solid-phase microextraction. *Anal. Chem.* **1993**, *65*, 1843−1852.

(12) Pawliszyn, J. *Solid-Phase Micro-Extraction: Theory and Practice*; Wiley-VCH: New York, 1997.

(13) Mestres, M.; Marti, M. P.; Busto, O.; Guasch, J. Simultaneous analysis of thiols, sulfides and disulfides in wine aroma by headspace solid-phase micro-extraction gas chromatography. *J. Chromatogr. A* **1999**, *849*, 293−297.

(14) Scarlata, C. J.; Ebeler, S. E. Headspace solid-phase micro extraction for the analysis of dimethyl sulfide in beer. *J. Agric. Food Chem.* **1999**, *47*, 2505−2508.

(15) Whitesides, G. M.; Houk, J.; Patterson, M. A. K. Acitvation parameters for thiolate−disulfide interchange reactions in aqueous solution. *J. Org. Chem.* **1983**, *48*, 112−115.

(16) Damodaran, S. Amino acids, peptides and proteins. In *Food Chemistry*; Fennema, O. R., Ed.; Marcel Dekker: New York, 1996; pp 321−429.

(17) Jocelyn, P. C. *Biochemistry of the SH group*; Academic Press: London, 1972.

(18) Chen, X.; Schofield, J. D. Determination of protein-glutathione mixed disulfides in wheat flour. *J. Agric. Food Chem.* **1995**, *43*, 2362−2368.

(19) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

(20) Sedlak, J.; Lindsay, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **1968**, *25*, 192−205.

(21) Onda, M.; Tatsumi, E.; Takahashi, N.; Hirose, M. Refolding process of ovalbumin from urea-denatured state − evidence for the involvement of nonproductive side chain interactions in an early intermediate. *J. Biol. Chem.* **1997**, *272*, 3973−3979.

Received for review January 18, 2001. Revised manuscript received June 21, 2001. Accepted June 22, 2001. This work was funded by the Biotechnology and Biological Sciences Research Council and Campden and Chorleywood Food Research Association.

JF0100797