Effect of Protein Glycation on Subsequent Volatile Formation†

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Lysozyme was incubated with glucose at 25 °C and either 12%, 65%, or 76% relative humidity (RH) for 30 days or at 50 °C and 65% RH for 3 days. The volatiles generated by the subsequent heating of the glycated samples and their individual polymeric components were trapped and analyzed by gas chromatography/mass spectrometry. The glycated lysozyme incubated at 25 °C showed qualitative differences in volatiles generated at different relative humidities. The sample incubated at 50 °C generated more glucose-derived volatiles than the 25 °C samples. Different volatiles were generated from each of the polymeric components isolated from the 50 °C sample of glycated lysozyme. We have demonstrated that storage conditions have an effect on the nature of volatiles generated upon subsequent heating.

INTRODUCTION

In past years, hundreds of flavor compounds have been identified in various heat-treated and/or processed foods. Many of these volatiles are formed in a series of reactions, known as the Maillard reaction, that occurs when amino acids are heated in the presence of reducing sugars (Maillard, 1911, 1912). The initial product is a labile Schiff base derivative of the amino moiety, which quickly isomerizes to the more stable ketoseamine adduct via Amadori rearrangement (Kuhn and Weygand, 1937). Hodge (1953) depicted a network of reactions that take place to form Amadori rearrangement products, flavors, and colors. In addition to color and flavor formation, protein polymerization occurs. Polymerization of long-lived proteins in vivo has been studied extensively (Stevens et al., 1980–81; Bunn, 1981; Kohn et al., 1984; Bucala et al., 1984; Monnier and Cerami, 1983; Eble et al., 1983; Watkins et al., 1985). It has been determined that these reactions occur as part of the normal aging process and are accelerated in people with diabetes.

Much research has been conducted in the determination of the volatiles generated from the thermal decomposition of Amadori products formed by the reaction of sugars and different amino acids (Mills and Hodge, 1976; Birch et al., 1980; Vernin et al., 1988). To the best of our knowledge, however, there has been no research which determined what volatiles are formed from the subsequent heating of the Amadori products of protein–sugar interactions or of the polymers formed during these interactions. Our goal, therefore, was to identify the volatiles generated from these interactions. In addition, we were interested in determining what effects, if any, were brought about by temperature, relative humidity, and time.

EXPERIMENTAL PROCEDURES

Chemicals. Lithium chloride, cobalt chloride, sodium chloride, potassium iodide, acetic acid, glycerol, glucose, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane hydrochloride, potassium hydroxide, and HPLC grade methanol and water were purchased from Fisher Scientific (Fair Lawn, NJ). Lysozyme (chicken egg white 3× crystallized, dialyzed, and lyophilized; catalogue no. L6876) and naphthalene were purchased from Sigma (St. Louis, MO). Bio-Gel P-100 polyacrylamide resin, sodium dodecyl sulfate (SDS), and bromophenol blue were purchased from Bio-Rad Laboratories (Richmond, CA). Tenax TA (60/80 mesh) adsorbent resin was purchased from Alltech (Deerfield, IL), and Carbotrap 20/40 mesh adsorbent resin was purchased from Supelco (Bellefonte, PA). Spectra Por 6 dialysis tubing with 3500 molecular weight cutoff was purchased from Spectrum (Los Angeles, CA). PhastGel high-density gels, SDS buffer strips, and Coomassie Blue R350 were purchased from LKB Pharmacia (Piscataway, NJ).

Sample Preparation. One gram (0.068 mM) of lysozyme and 300 mg (1.67 mM) of glucose were dissolved in 50 mL of HPLC grade water and lyophilized (Labconco, Kansas City, MO) overnight. Saturated salt solutions were made by dissolving either LiCl, $CoCl_2$, NaCl, or KI in 100 mL of boiling water. Relative humidity (RH) chambers were made by placing the salt solutions into 475-mL capacity jars fitted with airtight Teflon-lined caps. The LiCl, $CoCl_2$, and NaCl salt solutions were equilibrated at 25 °C to obtain 12, 65, and 76% RH, respectively. The KI salt solution was equilibrated at 50 °C in an oven to obtain 65% RH (Greenspan, 1977). After the chambers reached equilibrium, the lyophilized lysozyme/glucose samples were placed in glass jars within these chambers. The 25 °C samples were incubated for 30 days, and the 50 °C sample was incubated for 3 days. After incubation, the lysozyme/glucose samples were each placed in separate dialysis tubing and dialyzed for 24–48 h or until no unreacted glucose could be detected with Diastix 2803P reagent strips (Miles Laboratories, Elkhart, IN). The contents of the dialysis tubing were subsequently lyophilized overnight. Volatiles from native lysozyme (100 mg incubated at 50 °C, 65% RH, 3 days) as well as native lysozyme that came directly from the −20 °C freezer were also analyzed to determine what products were formed without glycation.

Polymer Fractionation. A gel permeation chromatography (GPC) column (68 cm × 25 cm i.d.) packed with 30 g of Bio-Gel P-100 polyacrylamide resin, was used to fractionate the 50 °C sample into its polymeric components. For each separation, 20 mg of the sample was dissolved in 1 mL of 0.1 N NaCl. The mixture was filtered on a 0.45-μm Zetapor disposable syringe filter (Cuno Inc., Meriden, CT) prior to being placed onto the column. The 0.1 N NaCl buffer solution flowed through the column at a flow rate of 18 mL/h by gravity. The eluent passed through a Varian (Sunnyvale, CA) 2050 variable-wavelength ultraviolet detector with the absorbance set at 280 nm. Data were recorded on a Spectra Physics (Sunnyvale, CA) 4290 integrator and PE Nelson (Cupertino, CA) analytical chromatography data system (version 5.1.5). The eluent was collected every 15 min using a Redifrac fraction collector (LKB Pharma-

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cia). Eluents corresponding to each of the different polymer fractions were pooled and dialyzed overnight to remove the salt buffer. The polymer fractions were then individually lyophilized overnight. This procedure was repeated several times. The samples were stored at −20 °C in a desiccator until further analysis.

Electrophoresis and Densitometry. A Phast System separation and control unit (LKB Pharmacia) was used to determine the purity of the materials separated by GPC. The separated materials were dissolved in a 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) (pH adjusted with potassium hydroxide) buffer containing 0.25 M SDS, and the solution was heated at 100 °C for 5 min before the addition of 0.01% bromophenol blue. This solution was serially diluted to give protein concentrations of 1000, 500, 250, 100, 50, and 25 ng/μL. The solutions were applied to PhastGel high-density gels on which PhastGel SDS buffer strips were placed. Initial separation conditions were 250 V, 10 mA, 3.0 W, and 60 Vh. Final separation conditions were 50 V, 0.1 mA, 0.5 W, and 0 Vh.

The gels were stained with Coomassie Blue using a Phast System development unit (LKB Pharmacia), and densitometric determinations of the dried gels were made using the LKB UltroScan XL densitometer (LKB Pharmacia).

Isolation of Volatiles. Each (100 mg) sample to be analyzed was placed into a 20 cm × 4 mm i.d. glass tube that was plugged with glass wool at both ends. The sample tube was connected in series, using a brass union, to a 10 cm × 4 mm i.d. tube containing Tenax TA and Carbotrap adsorbent resins. Air was flushed through the glycated lysozyme sample tube and then the adsorbent resin tube at a rate of 40 mL/min. The glycated lysozyme sample tube was wrapped with heating tape and heated to 155 °C. After 1 h, the adsorbent resin tube was disconnected and closed with brass end caps and stored at −20 °C until further analysis. Enriched polymer samples were analyzed only once because of the difficulty in obtaining sufficient material. For the other samples, duplicate analyses, using a different 100-mg aliquot of each sample, were performed.

GC/MS Analysis. A Model TD-1 short path thermal desorber (Scientific Instrument Services, Ringoes, NJ) directly interfaced to the GC on-column injector (held at 250 °C) was used to desorb the volatiles from the adsorbent resin tube at 255 °C for 10 min. The GC oven was packed with dry ice to maintain a temperature of −40 °C during desorption. Gas chromatography/mass spectrometry (GC/MS) analyses were performed on a Finnigan (San Jose, CA) MAT 8230 high-resolution mass spectrometer directly interfaced to a Varian 3400 capillary gas chromatograph. The samples were analyzed by electron ionization (EI) to obtain mass spectra for correlation with the computerized National Bureau of Standards (NBS) and Environmental Protection Agency–National Institutes of Health (EPA–NIH) data bases. A 60-m, 0.25-μm film thickness, 0.32-mm DB-1 fused silica capillary column (J&W Scientific, Folsom, CA) was temperature programmed from −40 to 40 °C at a rate of 10 °C/min and then to 280 °C at a rate of 4 °C/min. The mass spectrometer was scanned between masses 35 and 350 under the following conditions: source temperature, 250 °C; electron energy, 70 eV; filament current, 1 mA; scan rate, 1 s/decade; interscan time, 0.8 s. Data were acquired and processed using a Finnigan MAT SS300 data system.

Quantitation. Semiquantitative estimates for the concentrations of the individual volatiles were obtained by peak height comparisons to the internal standard, naphthalene, 10 ng of which was injected onto the adsorbent resin tubes before desorption.

RESULTS AND DISCUSSION

Unincubated native lysozyme, after being held at −20 °C in a desiccator, was heated to 155 °C for 1 h and generated acetamide, acetic acid (an artifact arising from lysozyme purification), aminobenzonitrile, benzothiazole, dimethyl disulfide, indole, and phenol (Table I). Incubation of native lysozyme at the most severe conditions used (50 °C, 65% RH, for 3 days) prior to heating generated six additional volatiles (Table I). The formation of these additional compounds (benzoxazole, dimethyl trisulfide, isocyanobenzene, phenylethanone, propanoic acid, and sulfur dioxide) may be the result of protein oxidation during incubation. Fujimaki et al. (1972), using amino acid analysis, observed significant loss of threonine and histidine when lysozyme was heated at 150 °C for 20 min but little decomposition of the other amino acids. At 180 °C, significant losses of the other amino acids were observed. Using electron spin resonance, Hayase et al. (1975) demonstrated the formation of free radicals during the heating of lysozyme at 150–190 °C. Kato et al. (1971) studied the thermal degradation of amino acids at higher temperatures and observed that phenylalanine and tryptophan generated indole and that tyrosine produced phenol. The results of these previous investigations may provide an explanation for our findings of dimethyl disulfide and dimethyl trisulfide (oxidation reaction of methionine), sulfur dioxide (oxidation reaction of cysteine and/or cystine), indole (thermal degradation of phenylalanine or tryptophan), and phenol (thermal degradation of tyrosine).

Table I. Concentration[a] of Volatiles Formed When Unglycated Lysozyme Is Heated

volatile	unincubated[b]	incubated[c]
acetamide	10, 2	16, 17
acetic acid	>500, >500	>500, >500
aminobenzonitrile	7, 10	17, 140
benzothiazole	5, <1	3, 2
benzoxazole	ND	25, 22
dimethyl disulfide	2, 16	63, 22
dimethyl trisulfide	ND	42, 22
indole	3, 3	95, 134
isocyanobenzene	ND	2, 10
phenol	20, 5	20, 22
phenylethanone	ND	2, 9
propanoic acid	ND	7, 7
sulfur dioxide	ND	>500, >500

[a] In parts per million for duplicate runs. [b] Lysozyme stored at −20 °C in desiccator without glucose. [c] Lysozyme incubated at 50 °C, 65% RH, for 3 days without glucose. [d] ND, not detected.

The lysozyme–glucose mixtures incubated at 25 °C at 12%, 65%, and 76% RH for 30 days gave, after heating to 155 °C, a number of volatile products (Table II) in addition to those derived solely from incubated lysozyme. Benzaldehyde, dihydropyranone, dimethylpyrazine, methylpyrazine, pyrazine, and pyridine were formed in all cases. Acetylfuran, acetylpyrrole, benzenemethanol, ethylpyrazine, furanone, and furfural were produced from mixtures incubated at 65% or 76% RH but not at 12% RH. Hydroxymaltol and methylethylphenol were formed when the 76% RH sample but not the 65% RH sample was heated. Small amounts of ethenylpyrazine and pyridinecarbonitrile were detected when the 65% RH sample but not the 76% RH sample was heated, a result that may be due to the variability (due, in part, to the use of on-column instead of split–splitless injection with the thermal desorber as well as the nonhomogeneity of the samples) of our analytical procedures and/or the small quantities of these two volatiles generated. It is interesting to note that previous and on-going studies (Wu et al., 1991) have shown that only the lysine-1 of lysozyme is glycated during incubation of lysozyme–glucose mixtures at 25 °C at different RH values. It appears, therefore, that the additional volatiles generated at 65% and 76% RH arose from increased numbers of lysine-1 moieties becoming glycated, probably due to the increased mobility of the glucose.

Incubation of a glucose–lysozyme mixture at higher temperature (50 °C) even for a much shorter time (3 days) at the same relative humidity (65%) resulted in the formation of several additional volatiles upon subsequent heating. In addition, the 50 °C incubate gave increased amounts

Table II. Concentration of Volatiles Identified in Glycated Lysozyme Incubated at Different Temperatures and Relative Humidities[a]

volatile	30 days			3 days
	25 °C, 12% RH	25 °C, 65% RH	25 °C, 76% RH	50 °C, 65% RH
ethenylpyrazine	ND[b]	5, <1	ND	ND
pyridinecarbonitrile	ND	4, <1	ND	39, 7
hydroxymaltol	ND	ND	57, 42	29, 80
methylethylphenol	ND	ND	55, 40	ND
acetylfuran	ND	5, 3	75, 2	30, 32
acetylpyrrole	ND	3, 1	33, 3	43, 21
benzenemethanol	ND	3, 1	45, 1	13, 12
ethylpyrazine	ND	1, 2	89, 2	47, 41
furanone	ND	52, 143	181, 66	126, 133
furfural	ND	12, 13	269, 53	68, 32
benzaldehyde	261, 28	35, 16	144, 13	164, 44
dihydropyranone	266, 82	327, 72	309, 96	525, 176
dimethylpyrazine	149, 69	281, 166	202, 96	243, 82
methylpyrazine	108, 71	45, 85	58, 58	106, 41
pyrazine	57, 99	224, 213	164, 17	42, 36
pyridine	9, 2	153, 30	10, 15	39, 43
furancarbonitrile	ND	ND	ND	6, 2
furanmethanol	ND	ND	ND	103, 26
methylfuran	ND	ND	ND	6, 2
methylfurfural	ND	ND	ND	8, 2
methylpyrimidinone	ND	ND	ND	8, 2
pyrrolidinedione	ND	ND	ND	2, 1

[a] Quantities in parts per million for duplicate runs. [b] ND, not detected.

of most of those volatiles formed as a consequence of incubation at 25 °C. Fast atom bombardment mass spectrometry analysis of the chymotryptic peptides of the 50 °C incubate indicated that, in addition to lysine-1, at least three more lysine moieties had been glycated (Wu et al., 1991) and that some polymer formation had occurred.

Electrophoresis of the materials separated by gel permeation chromatography indicated that the fraction with a molecular weight approximately the same as that of native lysozyme (monomer fraction) was pure but that the dimer was contaminated with 16% monomer and 20% trimer. The trimer fraction contained 25% dimer and 28% tetramer. The volatiles generated by subsequent heating of the enriched polymeric fractions of the glucose–lysozyme mixture incubated at 50 °C, 65% RH, for 3 days are shown in Table III. Many additional volatiles (e.g., furanylbutanone, tetrahydromethylfuran, propylpyrrole) are now detectable because of enrichment of the minor polymeric components in the sample. In addition, several volatiles appear to form only after the dimer (dihydrodimethylfuran, ethylpyrazine, methylpyrrole, oxazole, and pyrazine) or trimer (dihydromethylfuran, dipropylamine, ethylethanamine, ethylmethylimidazoline, ethylhexanol, methylbutanal, methylcyclopentanone, methylfuran, methylpentenal, octylfuran, and pyridinecarbonitrile) is heated.

A large number of the identified compounds are sugar degradation products generally associated with Maillard reactions (Feather, 1989; Baltes et al., 1989), and it is obvious that they arise from glycated lysine moieties. For example, dicarbonyls formed as a result of degradation of Amadori compounds can cyclize to give the furan and pyran derivatives identified. In addition, the dicarbonyls are thought to be the cross-linking agents that give rise to polymerized lysozyme (Kato et al., 1987).

Stephenson and Clarke (1989) demonstrated that asparagine can become deamidated even at temperatures as low as room temperature. Robinson and Rudd (1974) found that glutamine can be deamidated at 37 °C. We believe that these reactions generate ammonia during the subsequent heating and, together with the normal conversion of Amadori products to dicarbonyl compounds, are the source of many of the nitrogen-containing materials identified.

Table III. Concentration of Volatiles Identified in Different Polymeric Fractions of Glycated Lysozyme Incubated at 50 °C[a]

volatile	monomer[b]	dimer[b]	trimer[b]
acetylfuran	18	ND[c]	ND
benzenemethanol	4	ND	ND
dimethylfuran	22	ND	ND
dimethylpyrazine	90	ND	ND
furancarbonitrile	8	ND	ND
furanmethanol	10	ND	ND
furanmethanol acetate	14	ND	ND
furanylbutanone	41	ND	ND
methylbutanol	34	ND	ND
methylpyrimidinone	2	ND	ND
propylpyrrole	29	ND	ND
pyridine	330	ND	ND
pyridinecarboxamide	102	ND	ND
pyrrolidinedione	5	ND	ND
tetrahydromethylfuran	24	ND	ND
trimethylcyclopentenone	27	ND	ND
dihydrodimethylfuran	ND	55	ND
ethylpyrazine	ND	33	ND
methylpyrrole	ND	9	ND
oxazole	ND	11	ND
pyrazine	ND	6	ND
dihydromethylfuran	ND	ND	12
dipropylamine	ND	ND	367
ethylethanamine	ND	ND	32
ethylhexanol	ND	ND	160
ethylmethylimidazoline	ND	ND	16
methylbutanal	ND	ND	11
methylcyclopentanone	ND	ND	218
methylfuran	ND	ND	7
methylpentenal	ND	ND	140
octylfuran	ND	ND	27
pyridinecarbonitrile	ND	ND	3
acetylpyrrole	134	8	ND
hydroxymaltol	84	82	ND
pentylfuran	ND	57	88
propylfuran	ND	546	155
pyrrole	ND	135	72
benzaldehyde	528	244	399
dihydropyranone	331	881	676
ethylpyrrole	25	30	178
furanone	57	44	33
furfural	248	64	317
methylfurfural	135	25	65
methylpyrazine	38	66	14

[a] Quantities in parts per million. [b] Monomer is 100% pure. Dimer is 16% monomer and 20% trimer. Trimer is 25% dimer and 28% tetramer. [c] ND, not detected.

In conclusion, we have demonstrated that incubation of a protein with a reducing sugar will create an environment for the formation of numerous volatile compounds when the mixture is subsequently heated. In addition, we have shown that different storage conditions will have a significant effect on the quantities and types of volatile compounds that are generated.

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LITERATURE CITED

Baltes, W.; Kunert-Kirchoff, J.; Reese, G. Model Reactions on Generation of Thermal Aroma Compounds. In *Thermal Generation of Aromas*; Parliment, T. H., McGorrin, R. J., Ho, C.-T., Eds.; ACS Symposium Series 409; American Chemical Society: Washington, DC, 1989; pp 143–155.

Birch, E. J.; Lelievre, J.; Richards, E. L. Thermal Analysis of 1-deoxy-1-glycino-D-fructose and 1-β-alanino-1-deoxy-D-fructose. *Carbohydr. Res.* **1980**, *83*, 263–272.

Bucala, R.; Model, P.; Cerami, A. Modification of DNA by Reducing Sugars: A Possible Mechanism for Nucleic Acid Aging and Age-Related Dysfunction in Gene Expression. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 105–109.

Bunn, H. F. Nonenzymatic Glycosylation of Protein: Relevance to Diabetes. *Am. J. Med.* **1981**, *70*, 325–330.

Eble, A. S.; Thorpe, S. R.; Baynes, J. W. Nonenzymatic Glucosylation and Glucose-Dependent Cross-linking of Protein. *J. Biol. Chem.* **1983**, *258*, 9406–9412.

Feather, M. S. Sugar-Derived Deoxy-Dicarbonyl Intermediates as Precursors of Food Flavors and Aromas. In *Thermal Generation of Aromas*; Parliment, T. H., McGorrin, R. J., Ho, C.-T., Eds.; ACS Symposium Series 409; American Chemical Society: Washington, DC, 1989; pp 209–216.

Fujimaki, M.; Kato, H.; Hayase, F. Studies on Roasting Changes of Proteins. Part I: Changes of Casein and Lysozyme During Roasting. *Agric. Biol. Chem.* **1972**, *36*, 416–425.

Greenspan, L. Humidity Fixed Points of Binary Saturated Aqueous Solutions. *J. Res. Natl. Bur. Stand.* **1977**, *81A*, 89–96.

Hayase, F.; Kato, H.; Fujimaki, M. Chemical Investigation on the Changes in Lysozyme During Heating. *Agric. Biol. Chem.* **1975**, *39*, 1255–1262.

Hodge, J. E. Chemistry of Browning Reactions in Model Systems. *J. Agric. Food Chem.* **1953**, *1*, 928–943.

Kato, S.; Kurata, T.; Fujimaki, M. Thermal Degradation of Aromatic Amino Acids. *Agric. Biol. Chem.* **1971**, *35*, 2106–2112.

Kato, H.; Cho, R. K.; Okitani, A.; Hayase, F. Responsibility of 3-Deoxyglucosone for the Glucose Induced Polymerization of Proteins. *Agric. Biol. Chem.* **1987**, *51*, 683–689.

Kohn, R. R.; Cerami, A.; Monnier, V. M. Collagen Aging *In Vitro* by Nonenzymatic Glycosylation and Browning. *Diabetes* **1984**, *33*, 57–59.

Kuhn, R.; Weygand, F. *Ber. Dtsch. Chem. Ges.* **1937**, *70*, 769–772.

Maillard, L. C. *C. R. Hebd. Seances Acad. Sci.* **1911**, *153*, 1078–1080.

Maillard, L. C. *C. R. Hebd. Seances Acad. Sci.* **1912**, *154*, 66–68.

Mills, F. D.; Hodge, J. E. Amadori Compounds: Vacuum Thermolysis of 1-deoxy-1-L-prolino-D-fructose. *Carbohydr. Res.* **1976**, *51*, 9–21.

Monnier, V. M.; Cerami, A. Detection of Nonenzymatic Browning Products in the Human Lens. *Biochim. Biophys. Acta* **1983**, *760*, 97–103.

Robinson, A. B.; Rudd, C. J. Deamidation of Glutaminyl and Asparaginyl Residues in Peptides and Proteins. *Curr. Top. Cell. Regul.* **1974**, *8*, 248–288.

Stephenson, R. C.; Clarke, S. Succinimide Formation from Aspartyl and Asparaginyl Peptides as a Model for the Spontaneous Degradation of Proteins. *J. Biol. Chem.* **1989**, *264*, 6164–6170.

Stevens, V. J.; Monnier, V. M.; Cerami, A. Hemeglobin Glycosylation as a Model for Modification of Other Proteins. *Tex. Rep. Biol. Med.* **1980–81**, *40*, 387–397.

Vernin, G.; Metzger, J.; Obretenov, T.; Suon, K. M.; Fraisse, D. GC/MS (EI,PCI,SIM)-Data Bank Analysis of Volatile Compounds Arising from Thermal Degradation of Glucose-Valine Amadori Intermediates. *Dev. Food Sci.* **1988**, *18*, 999–1028.

Watkins, N. G.; Thorpe, S. R.; Baynes, J. W. Glycation of Amino Groups in Protein. *J. Biol. Chem.* **1985**, *260*, 10629–10636.

Wu, H.; Hartman, T. G.; Govindarajan, S.; Kahn, P. C.; Ho, C. T.; Rosen, J. D. Glycation of Lysozyme in a Restricted Water Environment. *Proc. Natl. Sci. Counc., Repub. China* **1991**, *15B*, 140–146.

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