Effects of Reaction Conditions and Reactant Concentrations on Polymerization of Lysozyme Reacted with Peroxidizing Lipids

Jorge A. Funes, Ulrike Weiss,[1] and Marcus Karel*

Previous studies have shown that lysozyme undergoes deteriorative changes when exposed to peroxidizing lipids. In the present study we show the influence of concentration of protein and lipids and of the degree of unsaturation of the lipid on these changes. We also determined the effects of freeze-drying of aqueous emulsions containing lysozyme and methyl fatty acid esters. Lysozyme polymerization and loss of biological activity are promoted by higher protein–lipid concentration and higher degree of lipid unsaturation. Freeze-drying promotes protein polymerization in aqueous emulsions containing lysozyme and peroxidizing methyl fatty acid esters and decreases lipid hydroperoxide and malonaldehyde concentrations. These results suggest that reactant concentration induced by freeze-drying promotes hydroperoxide decomposition and facilitates free radical transfer reactions between lipids and proteins. However, freeze-drying does not affect the rate of loss of enzyme activity of the protein.

Exposure of proteins to peroxidizing lipids or their secondary breakdown products can produce changes in proteins, including loss of enzyme activity, polymerization, insolubilization, scission, and formation of lipid–protein complexes. This interaction is affected by several factors such as temperature, water activity, presence of catalysts, and reactant concentration. These changes may not only seriously decrease the nutritional and organoleptic quality of a protein-containing food but also induce undesirable changes in living cells (Logani and Davies, 1980; McCay, 1981).

Protein–peroxidizing lipid interaction can take place mainly through two basic mechanisms (Karel, 1977): (1) protein–amino condensation reactions involving lipid peroxidation breakdown products such as malonaldehyde and protein amino groups; (2) reaction of proteins with lipid oxidation products (lipid free radicals, hydroperoxides, and volatile secondary products) with resulting formation of protein-centered free radicals.

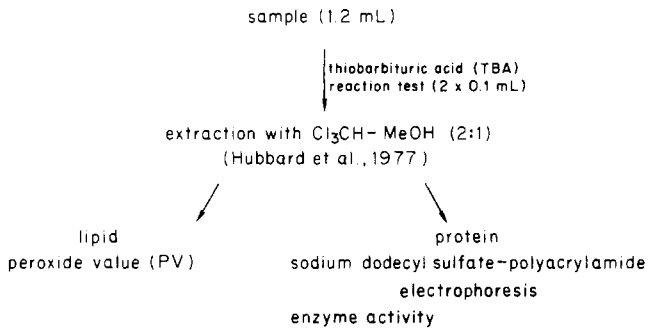
In the past we have studied the formation of protein-free radicals (Schaich and Karel, 1975; Funes et al., 1980; Funes and Karel, 1981) and their subsequent interaction with lipids achieved by lyophilization and adjustment of water activity under conditions of low and intermediate water activity. In later studies using more dilute aqueous systems we observed effects that apparently were due to inclusion of a freeze-drying step after reaction in aqueous emulsions. In the present study we describe results showing the effects of postincubation freeze-drying of aqueous emulsions containing hen egg lysozyme (LYS) and methyl fatty acid esters [methyl oleate (O), methyl linoleate (L), and methyl linolenate (Ln)]. We also show the influence of protein–lipid concentration and the degree of lipid unsaturation on the protein–peroxidizing lipid interaction.

MATERIALS AND METHODS

Experiments were carried out with aqueous emulsions of LYS and methyl fatty acid esters in a weight ratio of 1:1. Systems with two protein concentrations were prepared: system 10X consisting of 5.0% LYS and 5.0% lipid and system X consisting of 0.5% LYS and 0.5% lipid (weight/volume of water).

Preparation. For system 10X, the emulsions were prepared by dissolving 1.25 g of LYS (3× crystallized, ICN Nutritional Biochemicals, Cleveland, OH) in 25.0 mL of water; then 1.25 g of methyl fatty acid ester (O, L, or Ln, NU Check Preparations Elysee, MN) were added and emulsified in Sorvall Omni-mixer for 30 s and immersed in an ice–water bath. System X emulsions were prepared in a similar way by using 0.125 g of LYS and 0.125 g of lipid in 25.0 mL of water.

Oxidation was carried out in an open-air system consisting of 2.5 mL of emulsion in a 50-mL Erlenmeyer flask. This volume of sample formed a thin layer of emulsion, and we observed no effects attributable to limitation of oxygen diffusion into the sample. Experiments carried out with much larger volumes did, however, show difficulties. Evaporation was prevented by placing the Erlenmeyer flasks in a desiccator containing a large volume of water. Oxidation was carried out at 37 °C in the dark. Samples were analyzed every 1 or 2 days during a 10-day period. Each sample was divided into two 1.2-mL batches. One batch was subjected to the extraction and analysis procedure without freeze-drying, and the other batch was lyophilized in a Virtis laboratory freeze-dryer, with no external heating, at 100 μHg and at a condenser temperature of −25 °C and then diluted with 1.2 mL of water. The extraction and analyses were as follows:



Protein recovery after extraction was tested by the procedure of Lowry et al. (1951) using pure lysozyme as the standard.

Enzyme activity was determined by using the Worthington lysozyme reagent set as described by Funes et al. (1980). $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out by using the method of Weber and Osborn (1969) as modified by Pozzio and Pearson (1976). Sample preparation for electrophoresis was performed as described by Funes et al. (1980). Destained gels were scanned colorimetrically at 550 nm in a Hitachi Perkin-Elmer UV–visual gel scanner and integrated with a 339A Hewlett-Packard integrator.

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.
[1]Present address: 2000 Hamburg 50, West Germany.

0021-8561/82/1430-1204$01.25/0

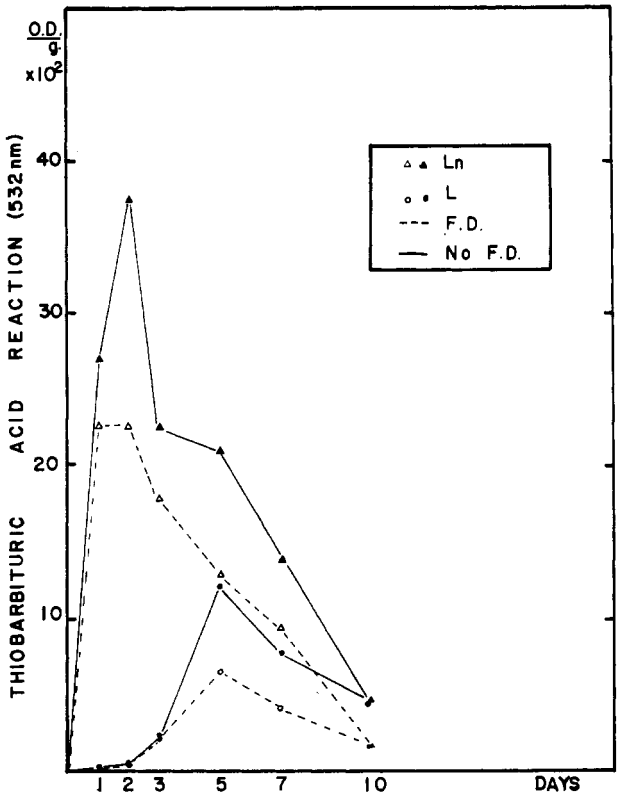


Figure 1. TBA values for 10X systems, with and without freeze-drying for Ln and L emulsions.

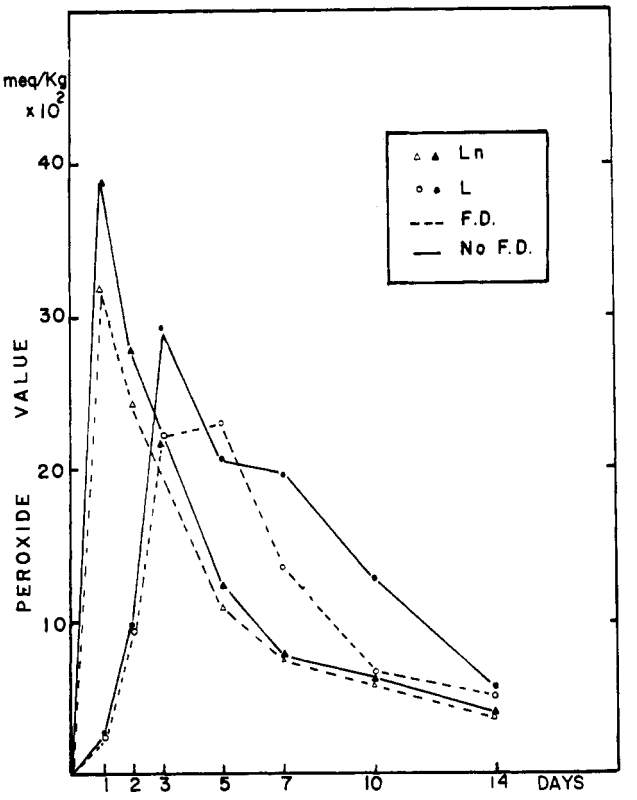


Figure 2. PV value for 10X systems with and without freeze-drying for Ln and L emulsions. O emulsions gave low PV after 5 days but less than 100 mequiv/kg.

Lipid oxidation was monitored by the TBA test as described by Asakawa and Matsushita (1980) and PV by iodometric titration (AOAC, 1970).

RESULTS

Lipid oxidation in the lysozyme–lipid emulsion was monitored by the TBA reaction and PV. Figure 1 shows the results of the TBA test for the concentrated emulsions (system 10X) and the effects of freeze drying. Similar results were obtained with system X. There is no TBA reaction with oxidation products of O as has been observed already by others (Pryor et al., 1976; Dahle et al., 1962). Figure 2 shows the lipid oxidation course as determined by the PV in the 10X system with and without freeze-drying. Results indicate that freeze-drying removes part of malonaldehyde and/or other volatile TBA-reactive products and also promotes lipid hydroperoxide decomposition.

The progress of lysozyme polymerization as a function of exposure time to peroxidizing lipid was monitored by disappearance of the lysozyme monomer and formation of lysozyme dimer fractions. The relative concentrations of these fractions are expressed as percent of total protein. They were estimated by scanning gels after electrophoretic separation and by determining the total soluble protein after lipid extraction. In calculating the percentages we assume that the optical density of each soluble fraction is equal to a constant multiplied by its weight.

Figure 3 shows the course of lysozyme dimerization and monomer disappearance in aqueous emulsions containing O, L, and Ln. Lysozyme polymerization in the aqueous emulsions is similar to that already observed for lysozyme exposed to volatile products of peroxidizing L (Funes et al., 1980). The monomer fraction continuously decreases, being transformed to higher molecular weight fractions. Also, the initial increase of the dimer fraction is followed by a decrease resulting from participation in the formation of higher molecular weight fractions. Figure 3 also shows

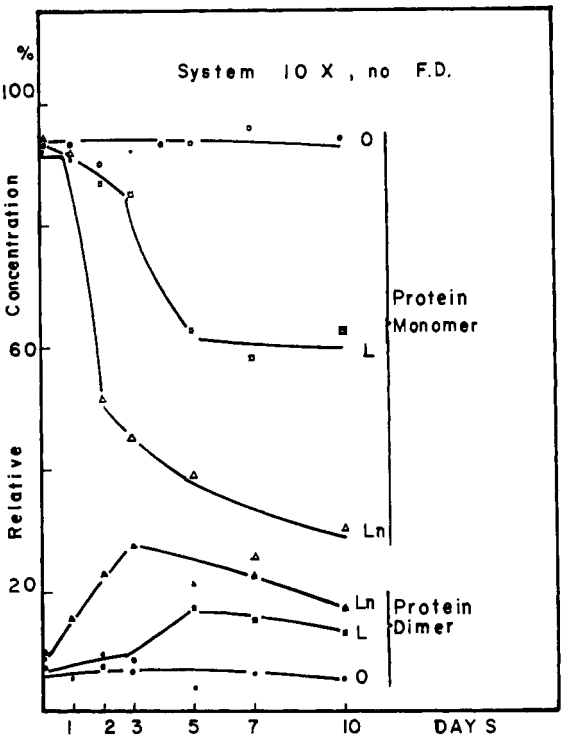


Figure 3. LYS polymerization as measured by dimer formation and monomer disappearance in LYS–lipid emulsion. Systems 10X (5.0% lysozyme–5.0% methyl fatty acid) were not freeze-dried.

that unsaturation of the lipid has an important effect on lysozyme polymerization. Peroxidizing Ln promotes a rapid lysozyme dimerization, while O has negligible effects. These differences are due to different oxidation rates.

Figure 4 shows the effects of concentration on the polymerization of lysozyme exposed to peroxidizing Ln.

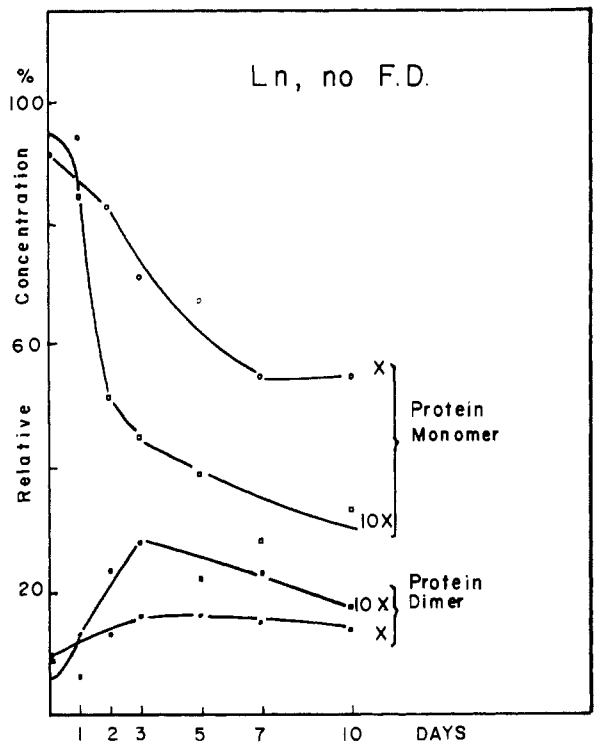


Figure 4. Comparison of LYS polymerization between system 10X (5.0% LYS–5.0% L) and system X (0.5% LYS–0.5% Ln) non-freeze-dried emulsions.

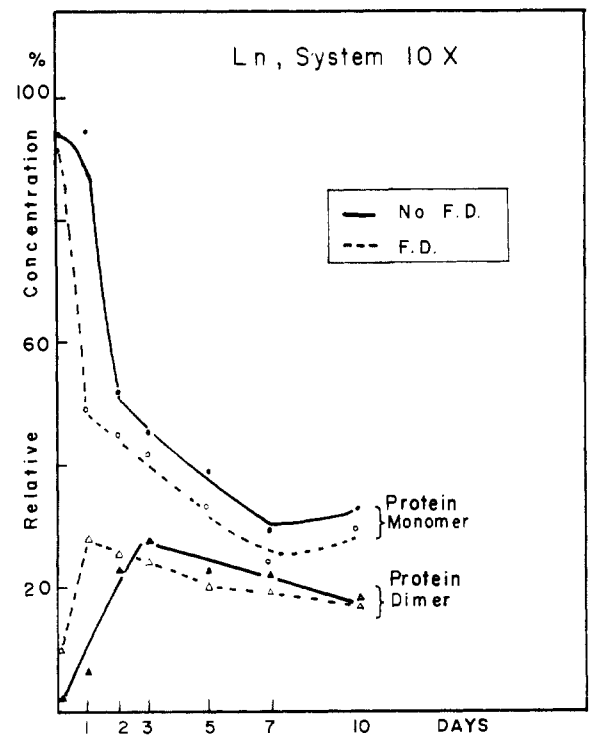


Figure 5. LYS cross-linking in LYS–Ln emulsions with and without freeze-drying.

System 10X is a 5.0% lysozyme–5.0% Ln emulsion in water while system X is 0.5% lysozyme–0.5% Ln. A 1:1 ratio of protein:lipid is maintained in both systems. A 10-fold increase in concentrations of both lipid and protein promotes lysozyme dimerization. Similar results were obtained in L-containing emulsions. No differences were detected in the case of O.

Figure 5 compares the course of cross-linking of the lysozyme–Ln system that was freeze-dried before lipid extraction with the same system that was not freeze-dried.

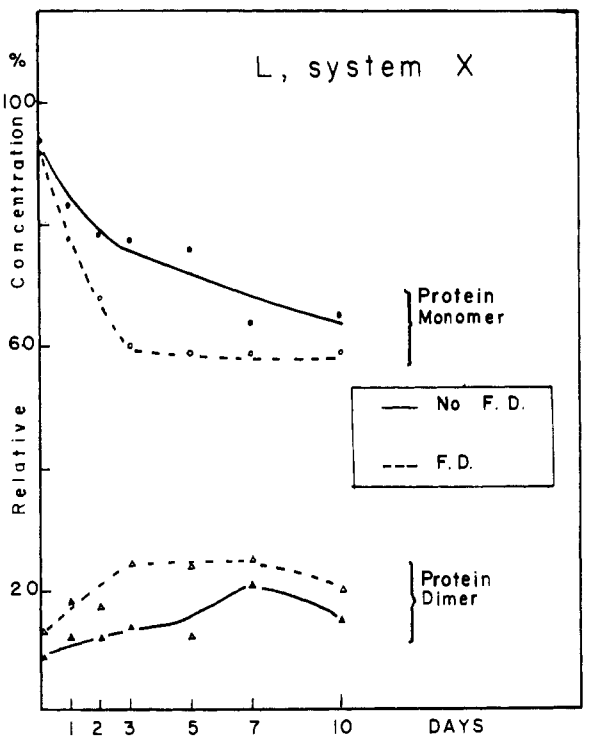


Figure 6. LYS cross-linking in LYS–L emulsions with and without freeze-drying.

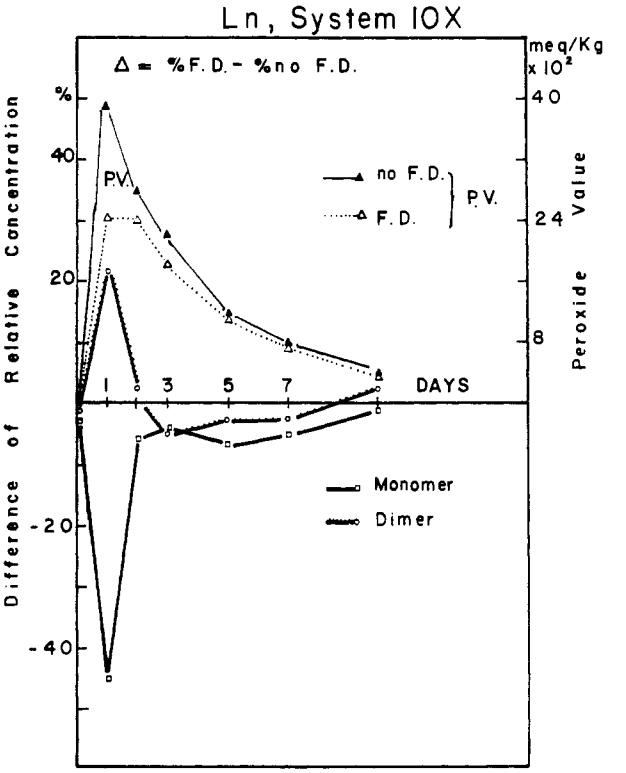


Figure 7. PV and percentage differences in the monomer and dimer fractions between the freeze-dried and non-freeze-dried LYS–Ln emulsions (system 10X).

Freeze-drying promotes polymerization, especially during the first 2 days, in the Ln system. In the case of L, similar differences in lysozyme polymerization due to freeze-drying are maximized at 3–5 days of oxidation (Figure 6).

The percentage differences in the monomer and dimer fractions between the freeze-dried and non-freeze-dried emulsions were calculated and plotted as a function of time. Figure 7 corresponds to the Ln emulsions and Figure

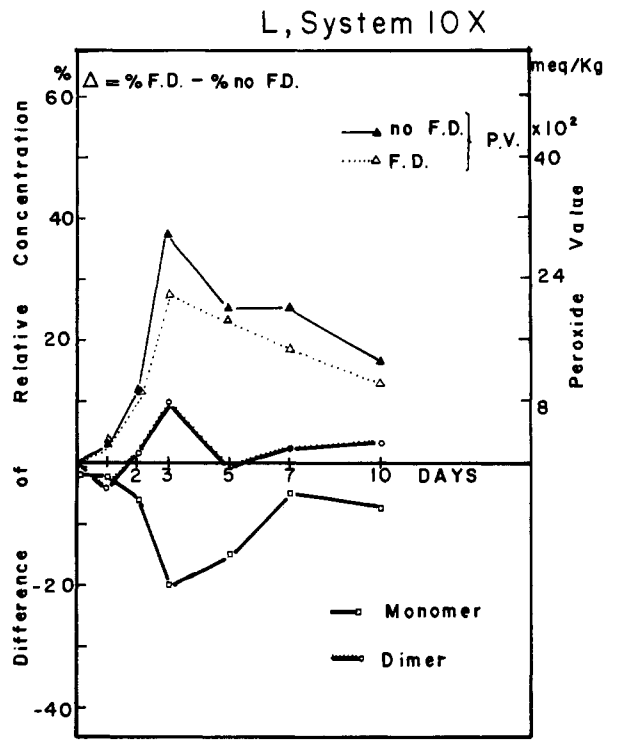


Figure 8. PV and percentage differences in the monomer and dimer fractions between the freeze-dried and non-freeze-dried LYS–L emulsions (system 10X).

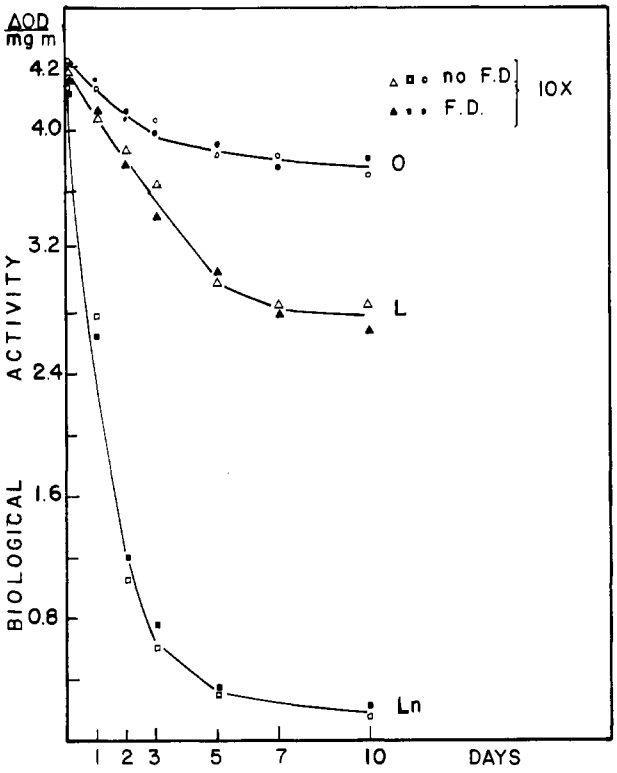


Figure 9. Loss of LYS biological activity upon exposition of peroxidizing methyl fatty acids in aqueous emulsions (system 10X).

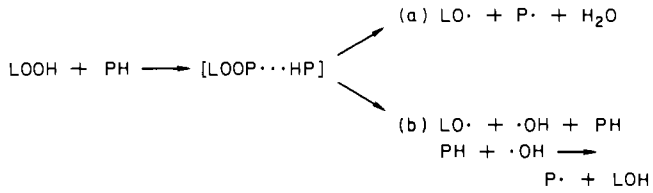
8 to the L system. These figures also show the lipid hydroperoxide concentration as determined by PV. The maximum differences in the disappearance of monomer and formation of dimer coincide with the maximum concentration of lipid hydroperoxides, suggesting that protein polymerization is promoted by an acceleration of lipid hydroperoxide decomposition by freeze-drying.

Control experiments were carried out in which extraction of peroxidizing lipids preceded freeze-drying of the aqueous phase containing lysozyme. In this case, freeze-drying did not increase the extent of the dimerization of lysozyme. This suggests that the presence of peroxidizing lipids during freeze-drying is necessary to induce lysozyme polymerization.

Figure 9 shows enzyme activity of lysozyme as a function of incubation time for the different methyl fatty acid esters. The results are expressed as the difference in optical density of the *Micrococcus lysodeikticus* suspension per milligram of lysozyme per minute. The degree of lipid unsaturation promotes differences in the rate of loss of biological activity, although we were not able to detect differences due to the freeze-drying itself.

DISCUSSION

Previous studies demonstrated that free radicals are involved in various lipid oxidation initiated changes in proteins (Schaich and Karel, 1975; Kanner and Karel, 1976; Schaich, 1980) and that protein polymerization is one of these changes (Karel, 1977). It has been postulated (Schaich and Karel, 1975, 1976) that complex formation between radical-producing species and proteins may precede the transfer of free radicals from lipid to protein—this being the rate that limits radical transfer. The complex formation was proposed as an initiation–free radical reaction between lipids (LH) and proteins (PH)



and also the propagation reactions

$$LOO\cdot + PH \rightarrow P\cdot + LOOH$$

$$LO\cdot + PH \rightarrow P\cdot + LOH$$

The protein-centered radicals are considered to be primarily C· and S· (Schaich and Karel, 1975), but the possibility of peroxyprotein radicals cannot be excluded. We have already shown that LH can be the original unfragmented lipids or their breakdown products (Funes et al., 1980). Such a mechanism would be dependent upon the oxidizability of the lipid fraction, originating lipid hydroperoxides and lipid free radicals, on the lipid and protein concentrations, and on the lipid hydroperoxide concentration. Higher protein and lipid concentrations as well as an increase in degree of oxidation would increase the rate of the free radical transfer reaction and subsequent protein polymerization.

Our results show that such concentration dependence exists as either an increase in the oxidizability of the lipid (in our experiments L vs. O resulting in higher lipid free radical and lipid hydroperoxide concentration) or an increase in the protein–lipid concentration (system 10X vs. X) that promotes a higher degree of protein polymerization as measured by the disappearance of monomer and formation of dimer of lysozyme. Our results also suggest that a higher concentration of lipid hydroperoxides facilitates the formation of protein free radicals, which can be explained by an increase in the rate of formation of the proposed complex [LOOH···PH].

The freeze-drying promoted lysozyme polymerization in the presence of peroxidizing lipids can be explained on the basis of two concomitant events: (1) concentration of protein and lipids by elimination of the solvent, which

results in a higher rate of protein free radicals formation, as already mentioned; (2) lipid hydroperoxide decomposition either as a complex [LOOH···PH] or as LOOH; both decompositions would promote protein free radical formation and subsequent protein polymerization in accordance with the proposed mechanism; the decreases of the PV of the lysozyme–lipid emulsions subjected to freeze-drying support this hypothesis.

Lysozyme biological activity decreases after treatment with peroxidizing lipids. Previous works (Kanner and Karel, 1976; Funes et al., 1980) have shown a loss of such activity upon treatment with peroxidizing L or their breakdown volatile products. Our inability to detect changes due to freeze-drying—which induces a higher lysozyme polyermization—would indicate that such loss is not associated with cross-linking.

Practical consequences of this work are that (1) usual methods to measure lipid oxidation (such as PV and TBA reaction) in lipid-containing systems subjected to freeze-drying may show erroneously low values and (2) the freeze-drying process can affect the quality of food systems in which lipid oxidation has already been initiated.

LITERATURE CITED

AOAC (Association of Official Analytical Chemistry) "Official Methods of Analysis of the Association of Official Analytical Chemists"; Horowitz, W., Ed; AOAC: Washington, DC, 1970; Methods 28.023 and 20.030, pp 445–446.
Asakawa, T.; Matsushita, S. *Lipids* **1980**, *15*, 137.
Dahle, L. K.; Hill, E. G.; Holman, R. T. *Arch. Biochem. Biophys.* **1962**, *98*, 253.
Funes, J.; Karel, M. *Lipids* **1981**, *16*, 347.
Funes, J.; Yong, S.; Karel, M. *J. Agric. Food Chem.* **1980**, *28*, 794.
Hubbard, W. D.; Sheppard, A. J.; Newkirk, D. R.; Prosser, A. R.; Osgood, T. J. *J. Am. Oil Chem. Soc.* **1977**, *54*, 81.
Kanner, J.; Karel, M. *J. Agric. Food Chem.* **1976**, *24*, 468.
Karel, M. In "Biotechnological Applications of Proteins and Enzymes"; Bohak, Z.; Sharon, N., Eds.; Academic Press: New York, 1977.
Logani, M. K.; Davies, R. E. *Lipids*, **1980**, *15*, 485.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
McCay, P. B. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1981**, *40*, 173.
Pozzio, M. A.; Pearson, A. M. *Biochim. Biophys. Acta* **1976**, *490*, 27.
Pryor, W. A.; Stanley, J. P.; Blair, E. *Lipids* **1976**, *11*, 370.
Schaich, K. M. *CRC Crit. Rev. Food Sci. Nutr.* **1980**, *13*, 189.
Schaich, K. M.; Karel, M. *J. Food Sci.* **1975**, *40*, 456.
Schaich, K. M.; Karel, M. *Lipids* **1976**, *11*, 392.
Weber, K.; Osborn, M. *J. Biol. Chem.* **1969**, *244*, 4406.

Received for review April 23, 1982. Accepted July 19, 1982. This study was supported in part by a contract with the U.S. Army Research Office, No. DAAG29-81-K-0081, and by Deutsche Forschungsgemeinschaft, Bonn-Bad, Godesberg, Federal Republic of Germany, through a grant to U.W. J.A.F. was a Fellow of the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina.

Additional Aroma Components of Honeydew Melon

Ron G. Buttery,* Richard M. Seifert, Louisa C. Ling, Edwin L. Soderstrom, Joseph M. Ogawa, and Jean G. Turnbaugh

In relation to the attraction of certain insect pests the volatile components of honeydew melon have been reinvestigated. Volatiles were isolated both by Tenax adsorbent trapping and by vacuum steam distillation continuous extraction. Major aroma compounds identified that had not been previously reported in melons include (*Z*)-6-nonenyl acetate, (*Z*,*Z*)-3,6-nonadienyl acetate, (*Z*)-3-nonenyl acetate, 3-methyl-2-butenyl acetate, and ethyl (methylthio)acetate (CH_3SCH_2COOEt). Odor threshold determinations indicted that (*Z*)-6-nonenyl acetate could be an additional important contributor to the total aroma for humans.

It is known that certain insects such as *Drosophila* spp. and *Nitidulid* spp. carry spores of *Monilinia* spp. and *Rhizopus* spp. that can cause diseases of fruit and nut crops (Tate and Ogawa, 1975; Wilson and Ogawa, 1979). It was observed that if honeydew melons are available that the same insects are strongly attracted to the ripe melons. It was felt that an improved knowledge of the volatiles of honeydew melons could provide basic information useful in understanding which chemical compounds are involved in the attraction of the insects to the various fruits. Such information might also make possible the formulation of synthetic attractive mixtures potentially useful for the trapping of these insects for both population estimation and possible control.

The volatile components of muskmelon and watermelon have been studied by Kemp and co-workers (Kemp, 1975; Kemp et al., 1972a,b, 1973, 1974). The volatiles of these and other melons including honeydew melon have also been studied by Yabumoto and Jennings (1977) and Yabumoto et al. (1978). None of these earlier studies, however, particularly concentrated on honeydew melon volatiles, and it was felt that further studies, aimed only at honeydew melon volatiles, were desirable.

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710 (R.G.B., R.M.S., L.C.L., and J.G.T.), Stored Products Insect Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fresno, California 93727 (E.L.S.), and University of California, Davis, Davis, California 95616 (J.M.O.).

EXPERIMENTAL SECTION

Materials. Ripe fresh honeydew melons (*Cucumis inodorus* Naud.) were obtained from experimental fields at the University of California, Davis, and from local markets.

Authentic chemical compounds were generally obtained from reliable commercial sources or synthesized by established methods. (*Z*)-6-Nonenal was synthesized as

0021-8561/82/1430-1208$01.25/0