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Fukushima,Danji; Van Buren,J.P.

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1821 University Avenue, St. Paul, Minnesota 55104

Mechanisms of Protein Insolubilization during the Drying of Soy Milk. Role of Disulfide and Hydrophobic Bonds[1]

DANJI FUKUSHIMA[2] and J. VAN BUREN, New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y.

ABSTRACT

Quantitative studies of the insolubilization of soy milk (SM) during drying showed that polymerization took place through disulfide bonds, as indicated by the effects of sulfhydryl-blocking agents, and through hydrophobic bonds, as indicated by the effect of sodium dodecyl sulfate. SM (7% solids) had 2 to 3 X 10^{-5} M active free sulfhydryl (SH) groups exposed on the surface of the molecules after a short heating time, and these took part in polymerizations during the drying step leading to insolubilization of 35% of the total SM proteins. However, these exposed active SH groups could be inactivated by prolonged heating before drying, perhaps by the oxygen dissolved in the soy milk. On the other hand, the amounts of the proteins insolubilized through hydrophobic bonds increased evenly with the heating time before drying and reached a plateau where 40 to 50% of the total proteins were insolubilized through the hydrophobic bonds. Most of the experimental findings concerning the redispersibility of dried SM proteins could be reasonably explained through these mechanisms.

In the previous paper (1), physical and chemical processing factors affecting the redispersibility of dried soy milk (SM) proteins were examined. It was found that the heating of SM before drying had a great influence on the redispersibility after drying. Redispersibility decreased rapidly to reach a minimum after several minutes of heating and then increased again. This increase in the redispersibility was much larger at 120° than at 100°C. The initial decrease has been qualitatively related to formation of disulfide (SS) bonds (1).

This research deals with a quantitative analysis of the mechanisms of the insolubilization of the SM proteins during drying.

MATERIALS AND METHODS

Soy Milk

The freeze-dried raw SM was prepared in the way described previously (1) and was used as starting material throughout this work.

Heat-Treatment of Soy Milk

A 7.0% (w./v.) SM solution was prepared from the freeze-dried raw SM. Samples (15 ml.) of the resultant SM were put into a 20-ml. ampule which was sealed and then incubated in a constant-temperature oil bath. In the shaking experiments with O_2 and N_2 the air in the ampule was replaced by these gases. The shaking was

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[2]Present address: Central Research Institute, Kikkoman Shoyu Co., Noda-shi, Chiba-ken, Japan.

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continued for 4 min. All the heat-treated SM was diluted to 6.5% (w./v.) with water or a solution of SH-blocking reagents and kept for 2 hr. at room temperature before drying began. At this stage the protein was completely dispersible.

Drying and Measurement of Redispersibility

Drying and subsequent measurement of redispersibility were carried out by the method described in the previous paper (1).

Extraction of Proteins by Sodium Dodecyl Sulfate

Soy milk was heated at 100°C. for 20 min., and after it had cooled to room temperature N-ethylmaleimide (NEMI) was added in a final concentration of 2 X 10^{-3}M, and then the resultant mixture was dried. Next, 50 ml. of solution containing the levels of sodium dodecyl sulfate (SDS) indicated in the text was added: protein redispersibility was then measured. pH was adjusted with NaOH to the levels shown in Table I.

RESULTS

Effect of SH-Blocking Reagents

To determine quantitatively whether the free SH groups present in heated SM proteins were responsible for the insolubilization of the proteins during drying, SH-blocking reagents were added to SM heated to 100°C. for 20 min.; after drying, protein redispersibility was measured. NEMI and Na-*p*-chloromercuribenzoate (PCMB) were used as the SH-blocking reagents because of their high specificity and quantitative reaction with free SH groups. As shown in Fig. 1, the redispersibility increased sharply with addition of NEMI and PCMB and reached a plateau. It should be noted that there was a very definite break point with both reagents where the concentration was almost identical in both reagents. Direct determination of the amounts of these reagents reacting with SH was difficult, because of the high blank values found with soy milk. However, it is quite clear from this figure that the 2 to 3 X 10^{-4}M SH groups of the SM proteins heated at 100°C. for 20 min.

TABLE I. PROTEINS OF DRIED SOY MILK INSOLUBLE IN SOLUTIONS OF SODIUM DODECYL SULFATE AT DIFFERENT pH LEVELS[a]

Extracting Condition		Proteins Not Solubilized	
SDS (w./v.%)	pH	Against Total Proteins %	Against Proteins Insolubilized by Causes Other Than SS Bonds %
0	6.8	47	100
0	10.4	4	9
0.1	7.1	21	45
0.1	10.5	2	4
0.5	7.2	2	4
0.5	10.5	1	2

[a]Method is described in "Materials and Methods."

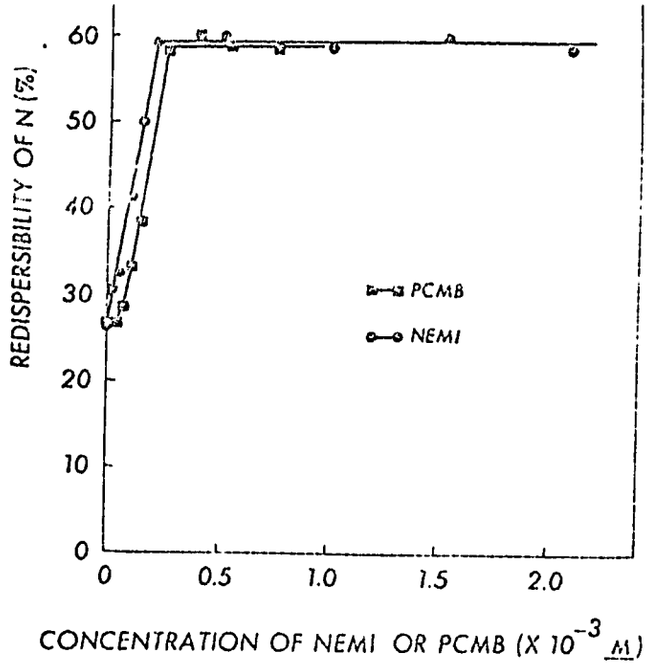

Fig. 1. Effect of concentration of NEMI and PCMB added to heated soy milk (SM) (100°C., 20 min.) before drying on redispersibility of dried SM proteins.

(the nitrogen content of SM, 0.456%) took part in the insolubilization, presumably through disulfide bond formation or SS exchange reactions during drying, or both.

Next, NEMI was added to SM after various heating times. The concentration of NEMI in the milk was 2×10^{-3}M, far in excess of the level shown in Fig. 1 to give maximum redispersibility. The shape of the redispersibility curves changed markedly upon this addition (Fig. 2). The large trough in each of the curves disappeared completely as a result of the blocking of free SH groups. The differences between the curves with and without addition of NEMI constitute the amounts of proteins insolubilized through SS polymerization. Furthermore, the difference between the 100% line and the curve obtained by the addition of NEMI constitutes the amount of proteins insolubilized by causes other than SS polymerization during the drying step. Figure 3 shows the amounts of the proteins which were insolubilized through each cause.

The amounts of the proteins insolubilized through SS polymerization increased abruptly as a result of heating and reached a maximum value (about 35%) after 5 to 10 min. of heating, both at 100° and at 120°C. After that the amount of insoluble protein decreased with the heating time, gradually at 100° and more rapidly at 120°C., finally going to zero. This insolubilization was comparable to that found (2) for insoluble disulfide polymers in isoelectric precipitates of dried soy protein. On the other hand, the amounts of protein insolubilized through causes other than SS polymerization increased evenly and reached a plateau after about 30 min. of

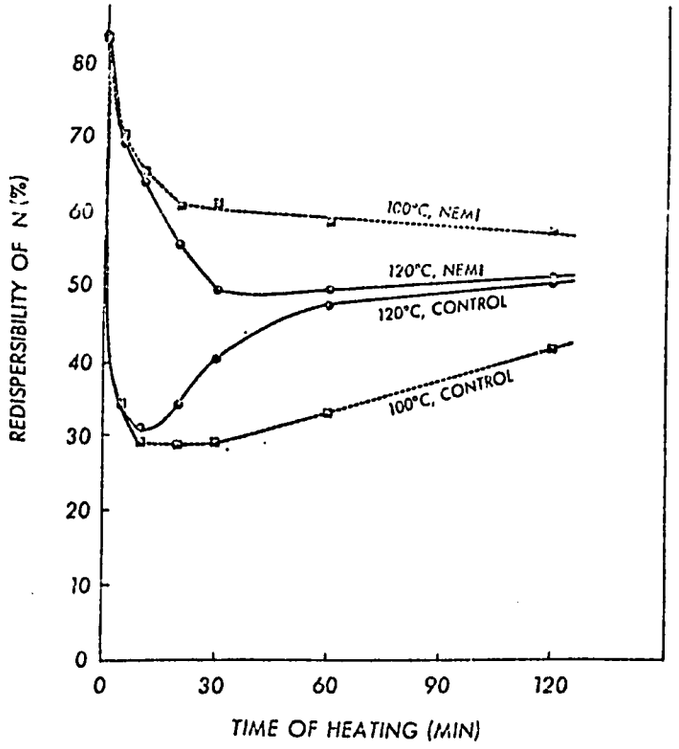

Fig. 2. Effect of NEMI added to heated SM before drying on redispersibility of dried SM proteins. Concentration of NEMI is 2×10^{-3}M.

heating, the values of which are 40% at 100° and about 50% at 120°C. heating. In the SM heated 5 to 10 min., when the insolubilization through SS polymerization during drying was at a maximum and consequently the redispersibility was at a minimum, about 35% of the total proteins were insolubilized through other causes, and 30% were not insolubilized during drying.

Effect of Presence of Oxygen during Heating

The decrease of SS polymerization insolubilization resulting from prolonged heating (Fig. 3) indicates that removal of the active free SH groups of the proteins took place during the heating. This SH removal was much more rapid at 120° than at 100°C. and might be due to oxidation by the oxygen solubilized in SM. In support of this, it was found that the redispersibility of dried SM proteins was greatly increased by contact with air or oxygen during heating (Figs. 4 and 5). This increased redispersibility was much higher at 120° than at 100°C. and higher in oxygen than in air. Shaking with nitrogen (Fig. 5) was not effective. With regard to SM shaken with oxygen during heating, the decreased insolubilization caused by the addition of NEMI was very small, indicating that free SH groups were inactivated very quickly in the presence of oxygen at the high temperature (Fig. 6).

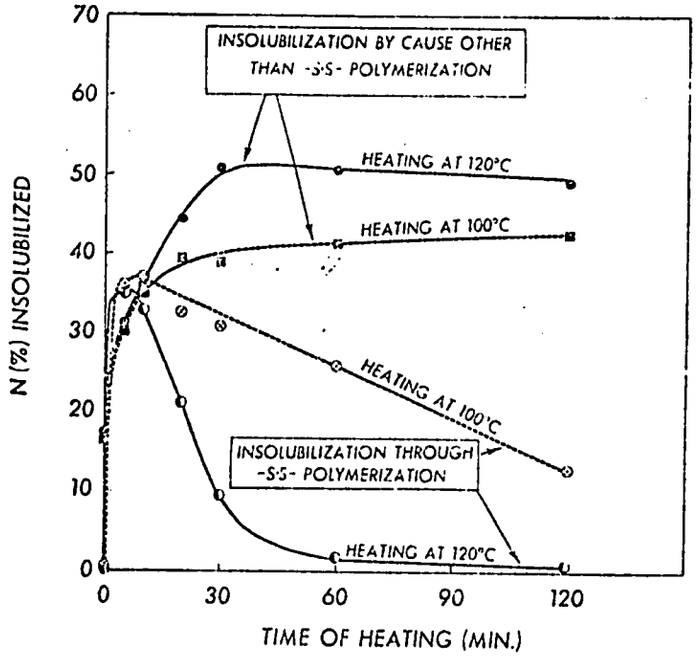

Fig. 3. Insolubilization of SM proteins during drying. (See explanation in text.)

Extraction of Insolubilized Proteins by Sodium Dodecyl Sulfate

Next, in order to know whether hydrophobic bonds are responsible for the insolubilization by causes other than SS polymerization, the solubility behavior of the SH-blocked dried SM proteins was tested with SDS, a hydrophobic bond-disrupting agent. Almost all the proteins in the SH-blocked dried SM (Table I) were solubilized by 0.5% SDS at neutral pH, indicating that the insolubilization by causes other than SS polymerization were largely the result of intermolecular polymerization through hydrophobic interactions between protein molecules or through other noncovalent bonds. Increased pH of the extraction media contributed to the degree of breakdown of the noncovalent bonds, but it was not always a necessary condition. This can be explained by electrostatic repulsion among the molecules, on which negative charge increases with increase in pH.

DISCUSSION

The heating and drying of soy milk gives rise to complicated differences in the solubility of the protein in the dried product. Short-term heating resulted in more insolubilization during the drying step than did long-term heating. These findings resemble those of Circle et al. (3), who found that the viscosity of soybean protein dispersions reached a maximum and then fell off sharply with more severe heat-treatment. An interpretation of the behavior of the total soybean protein on the basis of the known characteristics of 7S and 11S soy proteins would be

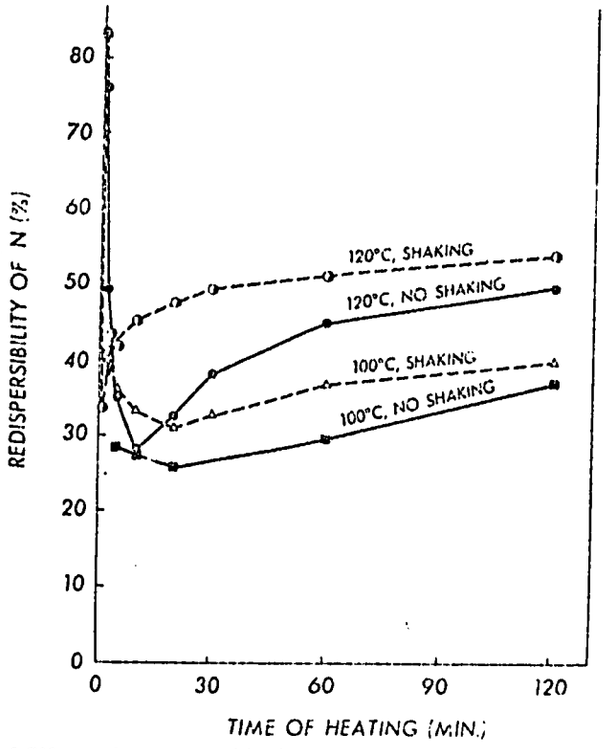

Fig. 4. Effect of SM protein contact with air during heating on redispersibility of dried SM proteins. Shaking was started at the same time as incubation, and continued for 4 min.

expected to have considerable validity, because these two fractions make up most of the proteins of soybeans. It was previously concluded that the three-dimensional structures of the major soybean protein (7S and 11S) have more than two free SH groups on their native molecular surface and are insolubilized through intermolecular disulfide bonds when they are brought to a precipitated state (2,4,5). The necessary and sufficient conditions for intermolecular SS polymerization are (a) the active free SH groups on the surface of a molecule and (b) the small distance between the molecules (5). When the proteins are kept in solution for a long time, the active SH groups located at the molecular surface are inactivated, perhaps changed to intramolecular disulfide bonds by the action involving the oxygen solubilized in the solution, because the long distance between the molecules reduces the opportunity for formation of intermolecular disulfide bonds. In the case of the unheated SM described in this paper, insolubilization of raw SM proteins during the drying step was around 16% of the total protein in both the presence or absence of NEMI.

This suggests that there were not significant amounts of active SH groups on the molecular surface of the unheated SM proteins. It is assumed that the active free SH groups which had been located on the molecular surface had been oxidized to intramolecular SS bonds, mainly by the air introduced into SM by vigorous shaking

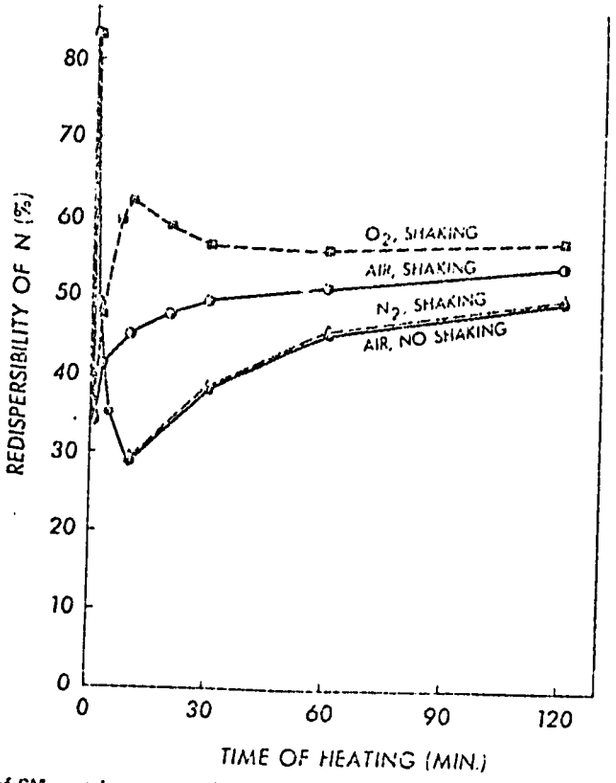

Fig. 5. Effect of SM protein contact with O_2, N_2, and air during heating at 120°C. Shaking was done in the same way as in Fig. 4.

during the initial extraction process, or by peroxides formed as a result of lipoxidase action; or they could already have formed intermolecular SS bonds to an extent not yet sufficient to cause insolubilization of protein. During the heating of SM the native three-dimensional structure of the proteins becomes disrupted, exposing SH groups formerly buried inside the molecules. When such protein molecules came close together during drying, the proteins had the opportunity to become insolubilized through formation of intermolecular disulfide bonds. However, when dilute SM was further heated, the active free SH groups, once exposed by heat-denaturation, apparently were gradually inactivated; this led to a decrease in the possibility of insolubilization through SS polymerization (Fig. 3).

The inactivated products formed from the free SH groups during heating might be intramolecular disulfide bonds, sulfenic acid (-SOH), sulfinic acid ($-SO_2H$), and sulfonic acid ($-SO_3H$) groups. The likelihood of intermolecular SS polymerization was low, since the dilute nature of the SM solution favored intramolecular reactions. Two possibilities for the oxygen donor are the oxygen dissolved in SM and the lipid peroxides (6,7) produced from the soybean oil. However, the latter possibility may be unimportant because the speed of the inactivation was not influenced substantially by the lack of lipids (Fig. 7; compare with Fig. 2).

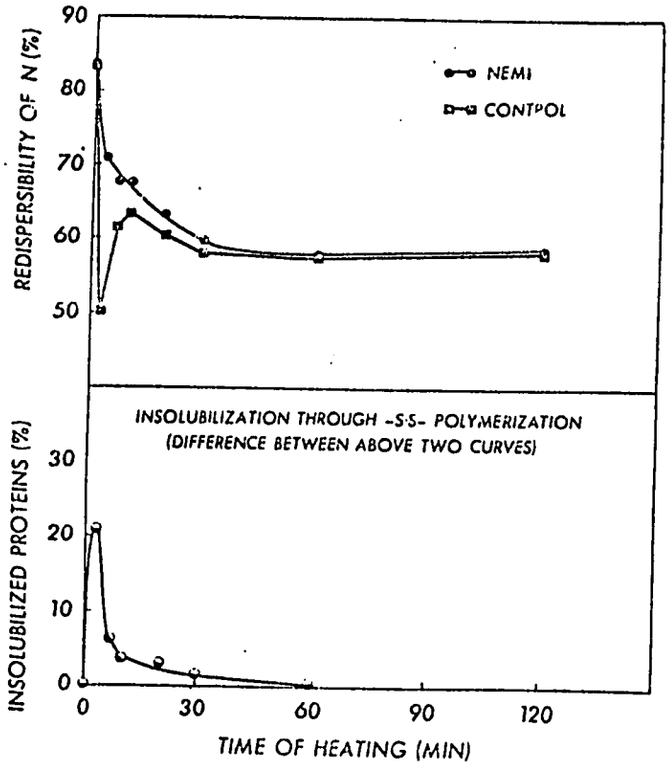

Fig. 6. Effect of NEMI added before drying on SM shaken with O_2 at 120°C. on redispersibility of dried SM proteins. Shaking was done as in Fig. 4. Concentration of NEMI was 2×10^{-3}M.

Therefore, the oxygen donor might be considered to have been the molecular oxygen dissolved in soy milk.

The possibility of insolubilization through covalent bonds other than the disulfide bond appeared low, since almost all the proteins in the SM dried with the addition of NEMI were solubilized by SDS, which does not split covalent bonds. Further, this solubility behavior with SDS suggests that the bonds responsible for the insolubilization through causes other than SS polymerization were hydrophobic bonds, because SDS has both hydrophobic and hydrophilic portions in its molecules and can break the hydrophobic bonds among the molecules by mechanisms described previously (8). When SM is not heated, the hydrophobic interaction among molecules occurs to only a small extent, because most of the hydrophobic groups of the native soybean proteins remain buried inside the molecules (9). In heated SM, however, the hydrophobic side chains of the major proteins can be exposed accompanying the breakdown of the three-dimensional structure of the molecule. The intermolecular hydrophobic interaction may then occur among the resultant hydrophobic residues when the molecules come closer as a result of the evaporation of water during drying. Longer time and elevation of temperature during the heating of SM before drying increased the exposure of the

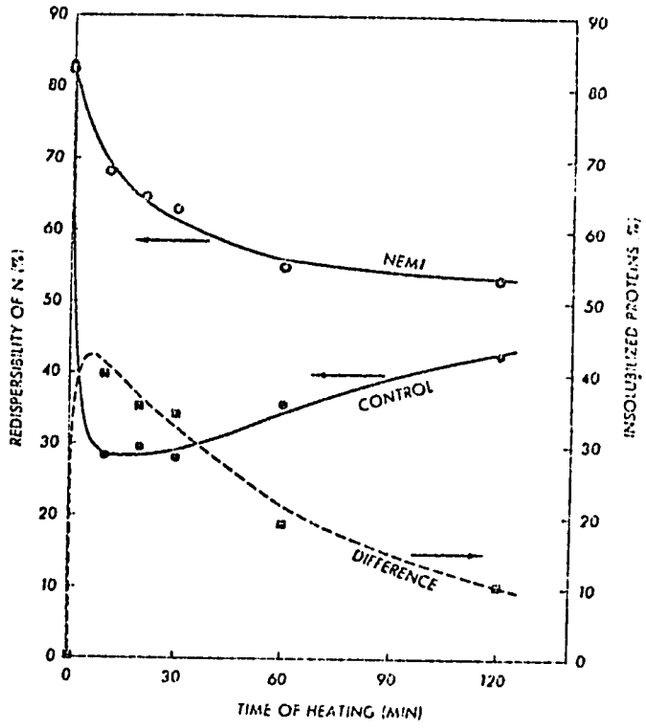

Fig. 7. Effect of addition of NEMI before drying on redispersibility of dried oil-free SM proteins. Oil-free SM was prepared from the soy flour defatted completely by ether. The curve "Difference" is the amounts of the proteins insolubilized through SS polymerization. Arrows show the direction of the horizontal axis. The concentration of NEMI was 2×10^{-3} M, heating temperature 100°C.

hydrophobic groups, leading additional amounts of proteins to become insoluble intermolecular polymers through hydrophobic interaction (Fig. 3). The pH in the solution is very important for formation of hydrophobic bonds among the molecules, because of electrostatic repulsion between molecules. With higher pH, each protein molecule increased its negative charge and consequently the mutual electrostatic repulsion increased. Thus, the formation of hydrophobic bonds was prevented or the formed hydrophobic bonds were broken, leading to an increase in the solubilized proteins (Figs. 3 and 4 in ref. 1) (Table I). It is concluded that the insolubilization of the SM proteins during drying occurs mainly through intermolecular SS polymerization and hydrophobic interaction. The complicated phenomena observed so far with regard to redispersibility of dried SM can be explained very reasonably by these mechanisms.

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