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THE ROLE OF THE INTERFACIAL PROTEIN FILM IN MEAT BATTER STABILIZATION

A. Gordon and S. Barbut

Animal and Poultry Science Department, University of Guelph, Guelph, Ontario, Canada, N1G 2W1.

Abstract

The microstructure of meat batters made with equal ionic strengths of NaCl, $MgCl_2$, $CaCl_2$ and KCl (IS=0.43) and a reduced-NaCl batter (IS=0.26) were examined by scanning and transmission electron microscopy. Micrographs revealed that fat globules with smooth and rough protein coats were present in all treatments. The roughly-coated globules were prevalent in the unstable batters. Pores were observed in the interfacial protein film (IPF) surrounding the globules and were more prevalent in the globules with rough protein coats. Fat was seen to exude from the pores in both types of globules. Fat globules were shown to be immobilized by the physical binding of their IPF to the protein matrix. Thread-like protein strands appeared to play a role in binding the smaller fat globules to the protein matrix. The IPF had a complex, multi-layered structure. Some of the larger globules had internal protein structures which were connected to the IPF and which partitioned and further stabilized the fat. The results indicate that fat emulsification and the interfacial film are important in preventing fat separation in meat batters.

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Direct inquiries to S. Barbut
Telephone number: 519 824 4120 ext. 3669

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Introduction

The solubilization of the salt soluble myofibrillar proteins in comminuted meat products is of great importance in preventing fat separation. These proteins are the major structural components of the comminuted meat matrix (Fukazawa et al., 1961a,b) and have also been shown to form an interfacial protein film (IPF) around fat globules (Swift et al., 1961; Galluzzo and Regenstein, 1978a; Jones and Mandigo, 1982). Finely comminuted meat products are a complex mixture of muscle tissue, fat particles, water, spices and solubilized proteins which are held together by a variety of attractive forces (Jones, 1984). These components are combined to form what has been referred to as either a meat emulsion (Theno and Schmidt, 1978) or a non-emulsion meat batter (Lee, 1985; Regenstein, 1988). A classical emulsion consists of two immiscible liquid phases, one of which is dispersed in the other in the form of a colloidal suspension (Kramlisch, 1977). In meat batters, fat globules constitute the dispersed phase (Swasdee et al., 1982) but are sometimes larger than the size required to form a true emulsion. Hence, there are currently two theories for explaining the stabilization of meat batters: a) the emulsion theory and b) the physical entrapment theory (Lee, 1985).

The emulsion theory suggests that the salt soluble proteins are drawn to, and concentrated at, fat globule surfaces thus forming a stabilizing membrane in the raw emulsion (Hansen, 1960). Jones (1984) proposed that undenatured myosin first forms a monomolecular layer around fat globules in uncooked emulsions to which other proteins are then bound by protein-protein interactions. This is possible because of the thin layer of melted fat which is believed to be formed on the surface of the fat globules as a result of localized frictional forces during comminution. The myosin molecule is believed to be oriented at the interface such that the heavy meromyosin (HMM) head is facing the hydrophobic phase and the light meromyosin (LMM) tail is towards the aqueous phase (Jones, 1984). This theory is supported by the relatively high surface hydrophobicity of the HMM S1 fragment of myosin (Borejdo, 1983). Galluzzo and Regenstein (1978a,b,c) and Schut (1978) have used model systems to show that myosin is adsorbed

to form a film during emulsification. Hence, it appears that myosin may act as an emulsifier even in its native state and form an interfacial film of defined viscoelastic and mechanical properties at the oil-in-water interface. These properties determine the stability of emulsions (Asghar et al., 1985). Consequently, the emulsifying action of myosin may be vital to the stabilization of the uncooked meat batter.

Borchert et al. (1967) showed the presence of the interfacial protein film (IPF) in cooked meat batters and suggested that it may play a role in stabilizing the product during cooking. Their study also revealed drastic changes in the microstructure of the protein matrix on cooking and the existence of small holes or 'pores' in the protein films around fat globules in the cooked product. Jones and Mandigo (1982) focused on these pores in their study of the mechanism of meat emulsion stabilization. They found numerous pores in the protein envelope surrounding larger fat globules as well as several small fat droplets in the vicinity of these pores. They therefore proposed that the pores play the role of a "pressure release" valve which allows the thermal expansion of fat during cooking without a collapse of the stabilizing IPF. They concluded that batter stability was related to the thickness of the IPF as well as the integrity and density of the protein matrix.

The physical entrapment theory of meat batter stability proposes that the fat phase is stabilized by physical entrapment within a gelled protein-water matrix. The myofibrillar proteins in the matrix of the uncooked batter are thought to exist in a sol form (Acton and Dick, 1984). Cooking causes the aggregation of the proteins to form a three-dimensional gel which physically traps the fat particles (Lee et al., 1981). This theory suggests that large amounts of undisrupted fat cells remain after the batter is made and therefore help to stabilize the raw batter. The theory was supported by the finding that non-protein emulsifiers decrease rather than increase meat batter stability (Meyer et al., 1964). The chemical and physical properties of fat as well as the size and distribution of fat particles have been shown to affect fat stabilization (Townsend et al., 1968; Smith et al., 1983; Lee, 1985). These findings suggest that the emulsification of fat by the myofibrillar proteins is incidental to fat stabilization by other mechanisms.

Deng et al. (1981) and Lee (1985) have reported that fat channel formation resulting from the melting of fat at high chopping temperatures was caused by the discontinuity of the protein matrix in unstable batters and led to fat and water loss. However, Deng et al. (1981) also noted that large amounts of broken interfacial film were found in unstable batters. Gordon and Barbut (1989) indicated that fat channel formation in $CaCl_2$ and $MgCl_2$ (ionic strength (IS)=0.43) destabilized batters was due to the formation of a weak interfacial film combined with the aggregation of the protein matrix. Fat and water lost from unstable batters were also found to be closely related (r=0.95). Hence, although the formation of a coherent protein matrix is important in the preparation of a stable meat batter, interactions between the encapsulated fat droplets, matrix proteins and water influence the stability of the system.

The results reported here are the second part of a study in which several chloride salts were used to produce stable and unstable meat batters (Gordon and Barbut, 1989). The specific objectives of this study were to investigate the microstructure of the IPF and explore its role in the stabilization of fat in meat batters.

Materials and Methods

Batter Preparation

Five mechanically deboned chicken meat (MDCM) batters were produced in three separate trials as previously described (Gordon and Barbut, 1989). Four different chloride salts (NaCl (2.5%), $MgCl_2$, $CaCl_2$, KCl) were used so as to give an ionic strength (IS) of 0.43. In addition, a reduced-NaCl batter (1.5%, IS=0.26) was also prepared. The 2.5% NaCl represents the most widely used level for comminuted products. The MDCM was obtained from a local processing plant and used to make 0.5 kg batches. The meat was frozen (-18°C) for one month prior to use. Proximate analysis of the raw meat as determined in duplicate (AOAC, 1980) was: 66.7% moisture, 16.1% fat, 14.3% protein and 1.1% ash. The batters contained 6.0% added water and the chloride salts (Fisher Co., Ont.), which were added (as solids) at the initial stages of comminution, varied between treatments. Batters were chopped for 4 min in a non-vacuum bowl cutter (Hobart, Model 84142, Troy, OH) at high speed; end point temperatures did not exceed 8°C. Small air bubbles were removed from the batters by tumbling in a pre-cooled table top vacuum tumbler (Lyco, Columbus, WI) for 30s at a pressure of 0.15 atm. Batters (34g) were weighed into 50ml plastic test tubes, centrifuged (Fisher Centrific, Fisher, Ont.) at low speed (600 G) for 5 min to remove air bubbles trapped during hand stuffing and cooked in a water bath at 0.66°C/min using a programmable controller (Haake PG20, Haake, Berlin, W. Germany) to an internal temperature of 69°C.

Electron Microscopy

For SEM 3 mm cubes were cut from the centre of cooked batters from each treatment and were broken such that the broken side would be used for SEM. The specimens were fixed in 2% glutaraldehyde/ 1% paraformaldehyde in 0.1M HEPES buffer, pH 6.0 (Sigma Chemical Co., St. Louis) for 2 hr, rinsed with buffer, post-fixed with 1% OsO_4 for 4 hr, rinsed, dehydrated through a graded series of ethanols, critical point dried, mounted on aluminum stubs with silver paint, sputter-coated with palladium/gold in a Hummer VII unit (Anatech Ltd., VA), and examined at 10 kV (Hitachi S-570 SEM, Tokyo, Japan) as described by Gordon and Barbut (1989). For TEM, alcohol dehydrated samples were infiltrated with Spurr's resin and cured (16 hr) in capsules at 60°C. Sections were cut, picked up on grids and stained for 10 min with uranyl acetate and 5 min with lead citrate and viewed at 60 kV on a JEOL JEM 100CX TEM.

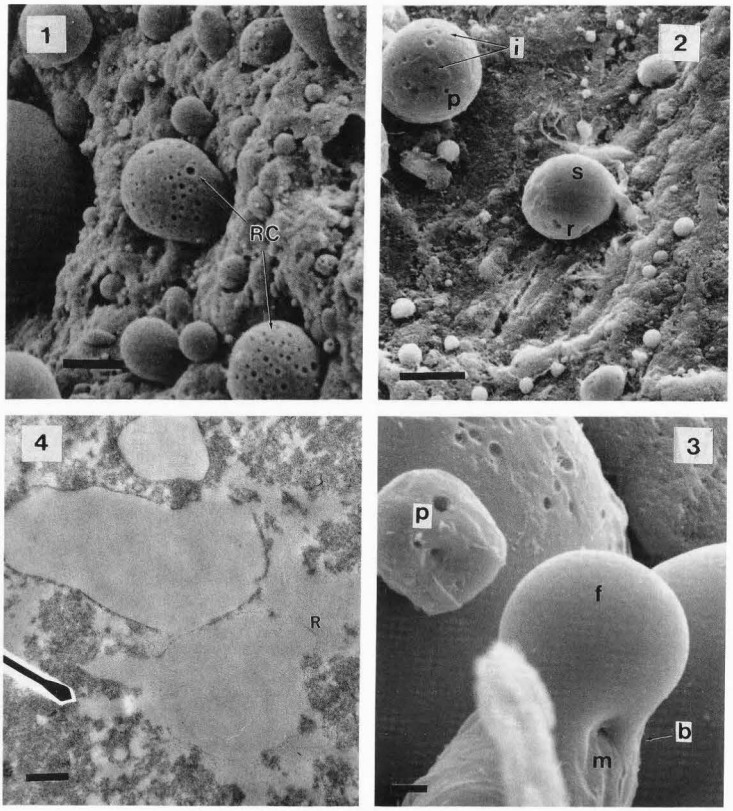


Figure 1. SEM of $CaCl_2$ treatment showing rough-coated (RC) globules with large pores (Bar=10μm).

Figure 2. SEM of KCl treatment showing small globules with smooth (s) and rough (r) protein coats. Note pores (p) concentrated in rough sections of coat and the indentations (i) (Bar=4μm).

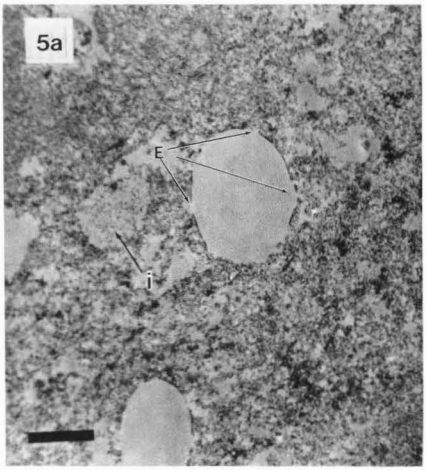
Figure 3. SEM of smooth and rough coated globules from the 2.5% NaCl treatment. Pores only visible in the rough coat. p - pore, F - fat globule, m - matrix, b -point of physical binding of fat globule to matrix (Bar=1μm).

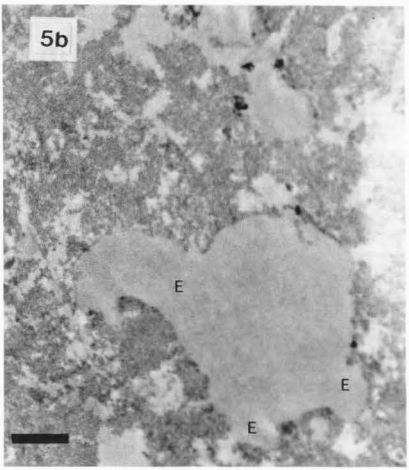
Figure 4. TEM of relatively stable, membrane enclosed globules adjacent to unstable globule with disrupted membrane ($CaCl_2$ treatment). Note thick coat which is continuous with the matrix around extended 'fingers'; r - protein film residue (Bar=0.5μm).

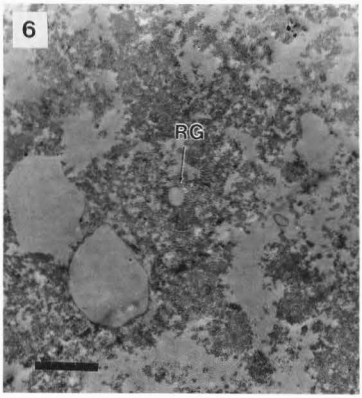
Results and Discussion

Fat Globule Morphology

The first part of this study was designed to examine the surface morphology of fat globules in both stable and unstable batters produced by using monovalent and divalent chloride salts respectively. A microscopical study of the five treatments revealed that two basic types of fat globules, smooth and rough, existed in meat batters







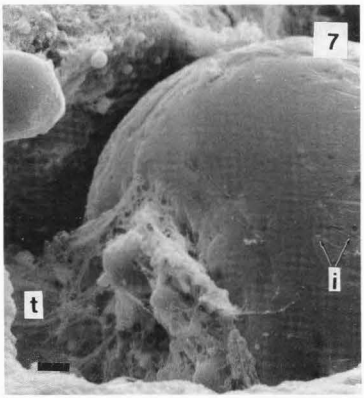


Figure 5. TEM of a) stable fat globules from the 1.5% NaCl treatment with relatively thick coats and evenly distributed pores; b) a globule ($MgCl_2$ treatment) with a thin protein coat in an unstable matrix showing multiple exudation of fat; I - internal structure; "E" - exudation (Bar=1μm).

Figure 6. TEM micrograph of a general field from the $CaCl_2$ treatment showing fat channels interconnected throughout the matrix. RG - round, stable globules (Bar=2μm)

Figure 7. SEM micrograph (2.5% NaCl treatment) of thread-like protein strands (t) bound to large fat globule and entrapping smaller globules. (Bar=2μm).

as was also described by Jones and Mandigo (1982). These two types were found in all treatments; however, the globules with rough surfaces were more prevalent in the unstable meat batters such as the

$CaCl_2$ treatment (Fig. 1). Pores were always present in the protein film of rough globules and were often relatively large in size. The rough area of the protein envelope was often not evenly distributed around the fat globule and there were globules in all treatments with both smooth and rough coats; the pores appeared to be concentrated in the rougher sections of the proteins coats (Figs. 2 and 3). The rough coat appeared to be due to thick, dense protein deposited unevenly around the globule. These deposits were continuous with the protein matrix as can be seen in a TEM cross-section (Fig. 4). Barbut (1988) also showed the presence of "rough" surfaces in batters prepared with different polyphosphates and NaCl. He suggested that the rough (wrinkled) appearance of the IPF was due to excessive fat loss from the globules during cooking. In this study, even globules which lost some of their fat retained their round shape (Figs. 1 and 4).

The smooth fat globules were prevalent in the more stable batters such as the 2.5% NaCl treatment. Basically, the smooth globules could be divided into two sub-groups. The first group was comprised of smooth globules which were relatively thickly coated with a few evenly distributed, tiny pores ranging from 0.01-0.1μm (Figs. 3 and 5a). The second group had globules with thin protein envelopes and larger pores (Fig. 5b). Generally, for smooth globules in all treatments, it appeared that the smaller, round globules had a relatively thick protein coat with few or no pores while larger round globules were thinly coated and had several pores. The irregular-shaped large globules tended to be thickly coated and had a rough protein envelope (Fig. 4). Lin and Zayas (1987) have also reported that large, irregular-shaped globules were thickly coated in frankfurters prepared with pre-emulsified fat. In the less stable batters ($MgCl_2$, Fig 4), protein envelopes ranged from fairly thick (>0.05μm) to almost indiscernible (<0.005μm).

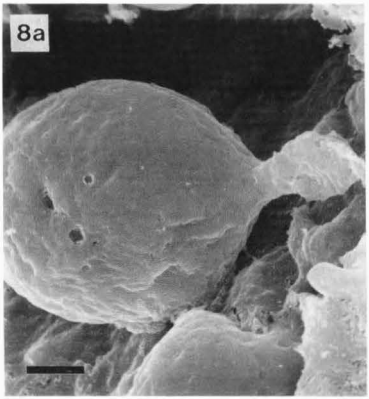
In a previous study, it was found that batter stability was affected by the type of chloride salt used; monovalent salts produced stable batters while $MgCl_2$ and $CaCl_2$ resulted in batter instability (Gordon and Barbut, 1989). In that paper it was suggested that the differences observed may have been due to differences in the quantity and type of protein extracted and we have recently found that protein extraction influences the morphology of fat globules in meat batters (Gordon and Barbut; in preparation). It therefore appears that the external morphology of fat globules is dependent on the type and amount of protein forming the IPF. The external morphology of fat globules is also affected by IPF thickness (Jones and Mandigo, 1982; Lin and Zayas, 1987). Jones and Mandigo (1982) have indicated that IPF thickness affects batter stability. Deng et al. (1981) also observed that IPF thickness was related to batter stability. Hence it appears that fat globule morphology in meat batters is a major determinant of batter stability.

Fat Stabilization

The thermal gelation properties of the protein matrix and the resulting physical entrapment of fat have been proposed as the major contributors to batter stability (Lee, 1985; Comer and Allan-Wojtas, 1988; Regenstein, 1988). However, while coalescence may be prevented by the physical restriction of fat by the protein sol in the uncooked state, it is difficult to see how fat not localized within a membrane could be stabilized by this entrapment mechanism during cooking. In the temperature range of 35-50°C, the fat would be liquid (Townsend et al., 1968) while most of the matrix proteins would not yet have begun to gel. Further, in the range of 50-70°C, the majority of the matrix proteins would have undergone thermal gelation (Acton and Dick, 1984) while the fat would still be in a molten state (Townsend et al., 1968). In both cases, the unlocalized large pools of fat should therefore spread freely throughout the aggregated matrix during cooking (unless they are confined by a cohesive matrix) resulting in the formation of a microstructure similar to that observed in the $CaCl_2$ treatment (Fig. 6). However, this is not the case in stable batters (Fig. 5a) and, even in unstable treatments ($CaCl_2$, Fig. 6), fat globules surrounded by a protein membrane remain stable during cooking. These observations support the idea of the importance of the IPF in fat stabilization during cooking.

In this study it was observed that many fat globules were physically restricted by being bound to the protein matrix. This may have resulted from protein-protein interactions between the IPF and the matrix proteins. TEM micrographs revealed that globules of various types and sizes showed continuity between their protein coats and the matrix at several points on their circumference (Figs. 5a and b). Theno and Schmidt (1978) and Gordon and Barbut (1990) have shown that physical binding of fat globules to the protein matrix does take place. Katsaras and Stenzel (1984) have also published micrographs which possibly show this phenomenon and Hermansson (1986) suggested that the proteins of the IPF may be part of the total protein network. It is therefore logical to assume that protein aggregation during cooking increases the immobilization of protein-coated fat globules by binding them to the matrix, thereby further stabilizing these globules and preventing coalescence. Thread-like protein strands were seen connecting the protein matrix and some fat globules, especially the smaller globules (Fig. 7). Hence, it is likely that the lacy structures often seen on the surface of fat globules in SEM micrographs (Theno and Schmidt, 1978; Jones and Mandigo, 1982; Gordon and Barbut, 1990) are remnants of this thread-like matrix protein bound to the IPF.

Jones and Mandigo (1982) proposed that fat was exuded through pores which developed at weak points in the protein envelope during cooking in response to internal pressure build-up. The present study provides some evidence of the existence of such a mechanism. Fat exudation was evident in all treatments. Exudation was observed with all types of globules but depended on fat globule size since very small globules (<1.0μm) which did not have pores did not show the phenomenon. Figure 8 shows exudation occurring in rough and smooth globules. Figure 8b shows multiple exudation which was occasionally observed, where even newly exuded globules themselves seemed to be in the process of fat release. No pores were visible on the surface of the exuding globule in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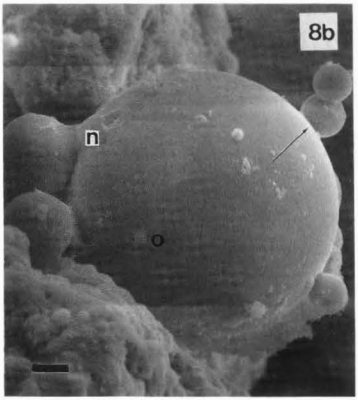
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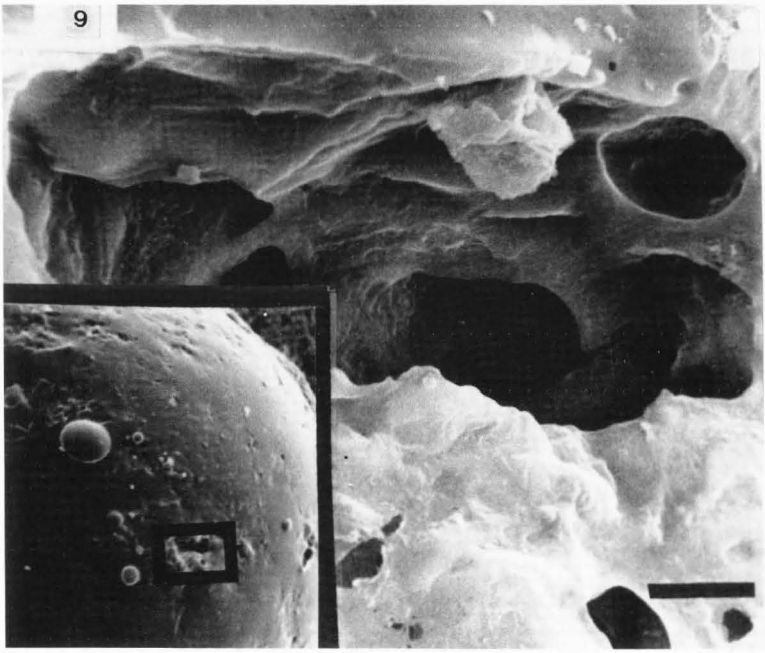
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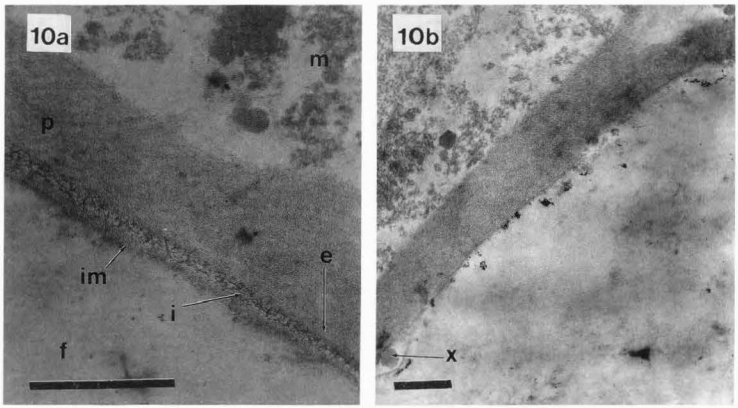


Figure 10. TEM of a) a thickly coated fat globule; b) a lower magnification of the IPF, both from the KCl treatment. m - matrix; p - thick, diffuse protein coat; e - external membrane; i - interconnecting diffuse region; im - internal membrane; f -internal fat; X - unidentified particle (Bar=1μm).

Fig. 8b. The protein coat around the exuded fat was continuous with that of the 'parent' globules (Figs. 5b and 8b). However, 'bumps' which may represent the early stages of exudation were evident in the protein coat of the fat globule (Fig. 8b).

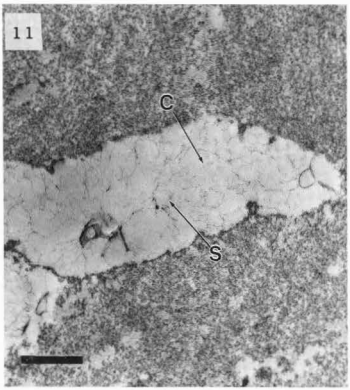
A possible mechanism for exudation and pore formation could be that during cooking, expanding fat pushes out the protein film at points of weakness to form round, stable appendages. These break off to form smaller round globules with the same type of protein envelope as the 'parent' globule (Fig. 8). This break leaves insufficient protein to properly seal the gap left in the parent globule, hence the formation of pores or indentations (Figs. 2 and 7). This phenomenon continues until smaller, more stable globules are formed or all the matrix and interfacial film proteins of the system gel as a result of cooking. The exuded fat associated with the formation of these pores or indentations was not always seen by SEM since it was probably retained as part of the fracture face on the other half of the specimen during fracturing for SEM preparation. However, these pockets of fat or globules are clearly evident in TEM preparations (Figs. 4 and 5b). It may be argued that the process described above represents fat coalescence and not exudation. However, while the more stable batters had globules which exhibited several small, uniform pockets of exuding fat (Fig. 5a), unstable emulsions contained globules which showed large exudations at weak points in their protein coats (Figs. 4 and 5b). These were more likely to form fat channels and facilitate coalescence (Gordon and Barbut, 1989). Fat coalescence can be distinguished from the exudation phenomenon in TEM by the lack of a defined spherical shape of the fat within the matrix (Koolmees et al., 1989), the existence of incomplete protein film residues within the fat (Comer and Allan-Wojtas, 1988; Gordon and Barbut, 1989) and the numerous inter-connections between fat pockets as can be seen in Fig. 6. The micrographic evidence presented here gives credence to the role of the IPF and the mechanism of exudation in meat batter stabilization.

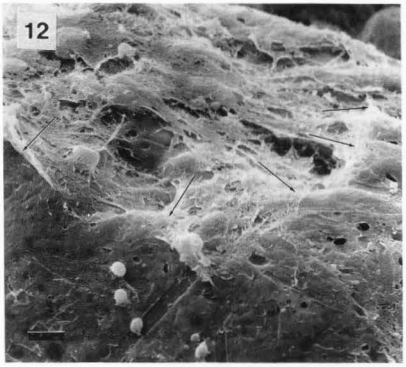
Structure of the Interfacial Protein Film and Interior of Fat Globules

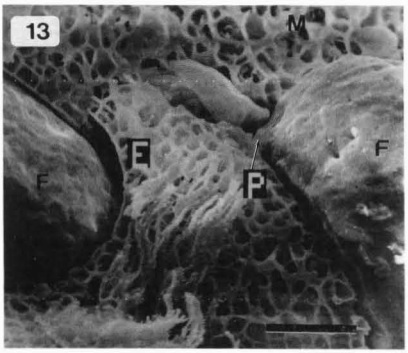
While the protein envelope which surrounds thinly coated fat globules is probably formed by the adsorption of a monomolecular layer of myosin (Jones, 1984), the protein film of more thickly coated fat globules appears to be more complex. The internal structure of a pore in a thickly coated fat globule appeared to consist of a complex, convoluted series of tunnels which seemed to extend into the globule (Fig. 9; showing the enlargement of the inset). Jones and Mandigo (1982) observed similar internal structures which they believed were internal pores within the main

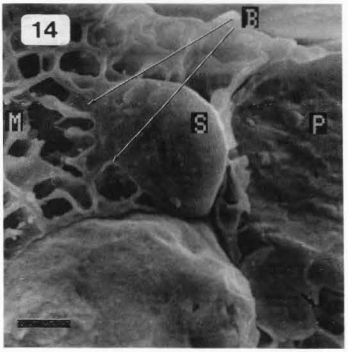
Figure 8. SEM micrograph of a) a rough globule from the 2.5% NaCl treatment with large pores in the process of exudation; b) a fat globule showing multiple exudation ($MgCl_2$ treatment). Note interconnecting 'neck'(n) of coat between parent and daughter globule. O - out-pushing ('bumps'); arrow - continuing process of exudation. (Bar=2μm).

Figure 9. SEM micrograph from $MgCl_2$ treatment showing close up of large pore in the surface of a rough globule (inset). (Bar=2μm).









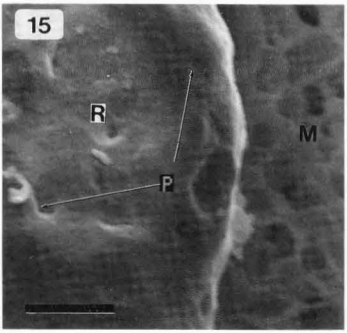


Figure 11. TEM of the compartmentalized internal structure of a membrane-bound fat globule (1.5% NaCl treatment). S - internal interconnected strands, C - compartment (Bar=1μm).

Figure 12. SEM micrograph of a fractured globule showing what appears to be lobes of protein coated fat further entrapped by mesh-like network of fibres ($CaCl_2$ treatment). Arrows-junction between protein envelope and fibres. (Bar=2μm).

Figure 13. Cold stage SEM (cryo SEM) micrograph of chicken meat batter made with 2.5% NaCl. E- physical entrapment within matrix, P- physical binding of fat to matrix, F- fat; M- matrix (Bar = 2 μm).

Figure 14. Cold stage SEM micrograph of chicken meat batter made with 2.5% NaCl. S- small fat globule, P- pore, M- matrix, B- physical binding (Bar = 2 μm).

Figure 15. Cryo SEM micrograph of chicken meat batter. R- rough coat, P- pore, M- matrix (Bar = 2 μm).

pores. They suggested that this could indicate the existence of an inner protein membrane and therefore that the IPF had a more complex structure than was previously thought to exist. We observed that the protein coat around thickly coated globules was multi-layered in nature (Fig. 10a). In this case, the IPF appeared to consist of four distinct layers. A thin internal layer coated the surface of the fat and was bound through a diffuse region to another layer of similar density. This in turn was bound to a very thick, relatively diffuse protein coat. The three internal layers of the protein coat seemed to form what may be a thermodynamically favoured lipid bilayer-type structure. Jones and Mandigo (1982) have proposed a similar structure for the IPF as is shown here. The two internal layers were shown to be separated by the interjunction of an unidentified particle (possibly bone from the MDCM) between them (Fig. 10b). The structure and formation of this membrane merits further investigation.

Some of the larger fat globules appeared to have a more complex internal structure than was originally thought. While small globules appeared to contain only fat, some of the larger globules contained internal structures of different densities and arrangements (Figs. 5a and 11). These might be the result of protein which coats internal fat, increasing the stability of the globule. This definitely appears to be the case in the compartmentalized structure evident in Figure 11. Lin and Zayas (1987) have also found internal structures assumed to be protein within large fat globules in frankfurters made with pre-emulsified fat. The internal appearance of a fractured fat globule from the $CaCl_2$ treatment revealed that its internal fat was partially separated into lobes but was essentially continuous throughout the globule (Fig. 12). The fat appeared to be enveloped in a fairly cohesive protein coat. The internal protein appears to be interlinked and this could possibly explain the convoluted sub-structure of the pore seen in Fig. 9. This protein may be the source of the internal channels within the pores which possibly form a route out of the globule for thermally expanding fat thereby facilitating exudation. A transverse section of the globule in Figure 12 would probably produce a structure similar to that shown if Figure 11. While the origins of these different levels of internal organization is unclear, the continuity of the IPF with the internal structure is clearly seen in Figures 5a and 12.

Conclusion

The dominant surface morphology of fat globules was found to be different between stable and unstable batters made with four chloride salts. These differences may be due to variations in the amount and type of protein forming the IPF. Fat was shown to exude through pores in the interfacial protein film in all treatments and was further stabilized by the binding of the IPF of fat globules to the protein matrix, thereby physically restricting the fat and preventing its coalescence in stable batters. The number and size of pores in the interfacial film appeared to depend on IPF thickness. The interfacial film has a complex, multi-layered structure which gives it stability and strength. The indications are that fat within some globules may be further stabilized by internal protein which interacts with the IPF. Further work is needed to more fully explore the functional significance of some of these observations. These findings indicate that fat emulsification and the interfacial protein film play a role in the production of stable meat batters.

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Discussion with Reviewers

A.M. Hermansson: In the preparation of the meat batters for electron microscopy the dehydration procedure is bound to affect the fat phase one way or another. Corroborative studies by freeze etching would be needed for the full evidence of fat exudation, the presence of pores and the distribution of the fat phase. Could the authors specifically comment upon the limitations of the preparation process?

Authors: All conventional electron microscopical (EM) preparation protocols involve a series of chemical and physical processes which can alter the microstructure of the specimen. Fixation with osmium tetroxide (OsO_4) can cause up to 30% specimen swelling, however this is often offset to some extent by the shrinkage which occurs later on in the preparation process (Hayat, 1981). Most of the structural modification of EM specimens occurs during alcoholic dehydration and critical point drying (CPD) for SEM and during embedding for TEM (Chabot, 1979; Dawes, 1988). Dehydration with alcohol can cause translocation of fat, fat leaching from the specimen and invariably, specimen shrinkage. This shrinkage is exacerbated by CPD for SEM and by shrinkage during embedding for TEM. These all produce artefacts which must be controlled by careful observation of sound EM preparation practices during specimen processing. However, their effects cannot be eliminated and together they affect the structure of the processed specimens which are viewed by EM. One must therefore be cognizant of the presence of these artefacts when interpreting electron micrographs.

The freeze-etching technique has not been utilized in our studies but we agree that it would be very useful for the kind of study undertaken in this paper. However, we have used cryo SEM (and surface etching by sublimation) to study meat batter systems. Figures 13 to 15 show some of the microstructural features described in the text as revealed by cryo SEM of chicken meat batters.

J.M. Regenstein: The whole issue of the amount of rancidity in the experimental samples and its implication for the interpretation of the data needs to be considered.

Authors: The extent of rancidity in the samples could affect the protein-lipid interactions. However, all treatments were made from the same source of meat so the variations in fat rancidity between treatments was controlled. In addition, many of these microstructural features have also been observed with other batters prepared from different meat and fat sources (unpublished data).

A.M. Hermansson: The fat observed in this study was part of chicken meat. Both the properties of the fat and the meat raw material may have a bearing on the final structure of a cooked meat system and it may be dangerous to generalize from studies made on one system. Have the authors studied the phenomenon of "fat exudation" or the nature of interfacial films of any other meat system?

F.W. Comer: In your formulations the only fat is coming from MDCM. Due to the recovery procedures for MDCM (mechanical shear and high pressure) it is unlikely that any cellular fat structure is retained, and in fact some protein coating may occur during mechanical deboning. Have you examined meat homogenates containing pork back fat or other adipose tissues? I would be interested to know whether the "speckled egg" pattern in Figure 1 has been observed in this type of meat homogenate.

Authors: In subsequent studies we have used a

chicken breast meat/pork back fat system to study the interactions involved in the stabilization of meat batters (unpublished data). The results of these studies have indicated that fat exudation and the multilayered IPF structure presented here also exists in these systems. We have not yet examined red meat systems but intend to do so.

C.M. Lee: All studies related to meat emulsion stability should include light microscopic data which would serve as supporting evidence to the EM data. Please comment.
Authors: We have used LM to examine the gross morphology sections from the same tissue blocks used for TEM sections. However, it was felt that because of the nature of this study, the resolution offered by light microscopy was not high enough to assist in clarifying the material under investigation.

J.M. Regenstein: Storage time of the meat batter will affect its properties, mainly leading to a stiffening of the material. This suggests that changes in the matrix may be more important than the authors identify and that the thin layer of liquid fat immediately after chopping may not be of fundamental importance. Please comment.
Authors: It was not our intention to suggest that changes in the protein matrix are not important. In fact, there can be no denying the vital role which protein gelation during cooking plays in the development of texture as well as water- and fat-binding. However, available research suggests that interfacial protein film (IPF) formation does take place in meat batters. We therefore believe that its role in batter stabilization should be considered.

J.M. Regenstein: Our work on timed emulsification and cream layer formation does not necessarily favour the emulsion theory. In fact later work with insoluble muscle systems would suggest that solubility and possibly the activity at the interface may not be the critical element. Please comment.
Authors: We are aware of this work (Gaska and Regenstein, 1982a,b; Perchonok and Regenstein, 1986a,b; Huber and Regenstein, 1988). We have also found that insoluble proteins may be important in fat binding in raw $CaCl_2$ batters (Gordon and Barbut, submitted). However, our work to date suggests that regardless of the origin of the proteins forming the protein film, the IPF works in conjunction with matrix protein gelation to stabilize raw and cooked batters. Our studies on $CaCl_2$ meat batters also suggest that the source of the proteins (soluble or insoluble) which form the IPF may be more important in determining fat stability during cooking than it does in the raw state.

F.W. Comer: It was clear from the first part of this study (Gordon and Barbut, 1989) that the major reason for differences in stability between treatments with various salts was the effect upon the protein matrix. The divalent cations produced "highly aggregated matrices" which resulted in large water losses. Differences in fat losses were relatively small. In this paper it is shown that the divalent ions produce thicker protein coating. Does this imply that more protein is extracted by the divalent salts? Intuitively, I would expect thicker protein coats to increase stability.
Authors: A recent study has shown that divalent chloride salts extracted less protein from meat than monovalent chloride salts (Gordon and Barbut, in preparation). Consequently, the thick protein coat in these batters cannot be due to soluble proteins. However, insoluble proteins are capable of participating in IPF formation (Schut, 1978; Gaska and Regenstein, 1982 a,b; Huber and Regenstein, 1988). Subsequent studies done in our laboratory have suggested that insoluble proteins do form an IPF around some fat globules in the divalent salt batters (Gordon and Barbut, unpublished). Because of the nature of these proteins, they appear to be distributed unevenly around the circumference of the globules (pointer, Fig. 4) resulting in weak (thin) areas at points on the circumference of the globules. These points were predisposed to rupture during cooking, thereby causing fat loss from the globule (Fig. 6). It should be noted that Jones and Mandigo (1982) also found that overly thick protein coats around fat globules reduced their stability.

J.M. Regenstein: Do rough globules have a protein envelope?
Authors: It appears that they do. However, this envelope tends to be of uneven thickness (see Figs. 4 and 6) and appears to be bound to the protein matrix and this may be what results in the "rough" appearance after fracturing and viewing by SEM (Figs 2 and 8a). Some globules had both rough and smooth areas (Figs 2 and 3) and this may represent differences on the circumference of the globule between areas with a defined IPF and those where the matrix proteins were directly in contact with the fat (See Fig 5a).

J.M. Regenstein: In the TEM, how do you decide if something is really a protein envelope around an oil droplet, i.e., I presume that means it serves as an interfacial film in part versus the fact that the matrix needs to terminate when it gets to a discontinuity. (Is it possible that the binding of the matrix to the fat is important, but that the traditional film formation is not?).
Authors: You have raised some very important points. In our TEM preparation procedure, secondary fixation with osmium tetroxide (OsO_4) was employed. Osmium tetroxide reacts mainly with the unsaturated fatty acids and, in addition to stabilizing the fat, it imparts greater electron density to areas where these fatty acids are concentrated. These unsaturated fatty acids would be concentrated on the outside of the fat globule where a thin film of liquid fat is believed to be located (Jones, 1984). The OsO_4 also produces acidic binding sites for the heavy metal ions (mainly the lead) which are later used to increase the contrast of the components within the sections (Dawes, 1988). In addition, OsO_4 also reacts with proteins, the extent of which depends (among other things) on protein conformation (Hayat, 1981). All the proteins within the system were exposed to OsO_4 but those at the interface would react differently from those in the matrix because they

assume different conformations (Graham and Phillips, 1979) or are different proteins (Gordon and Barbut, in preparation). Hence, all of these interactions result in an increased electron density on the circumference of fat globules when an IPF is formed which is distinct from cases where the matrix merely terminates next to a fat particle. Other researchers have shown that the use of OsO_4 improves the image of fat globule membranes in emulsions (Liboff et al., 1988).

The binding of the matrix to the fat is important and the great majority of fat particles within a meat batter are bound directly to the matrix (Fig. 13 and 14) in both raw and cooked batters. However, this direct binding appears to require the mediation of a protein coat which at least covers a part of the fat particle thereby allowing some level of immobilization of the fat once it is bound to the matrix. In recent work (Gordon and Barbut, in preparation) we have shown that it appears that the stability of $CaCl_2$ raw batters is due to the formation of an IPF around fat globules by the insoluble proteins which are also a part of the matrix. Earlier, Hermansson (1986) had suggested that the IPF proteins may be a part of the protein matrix. Consequently, even in a case where soluble proteins are not available for IPF formation, the insoluble (but surface-active) proteins such as actomyosin (and some myosin) appear to play a role in fat binding. It should be noted that whether soluble or insoluble proteins form the IPF, its binding to the matrix makes it an integral part of the protein gel network in meat batters.

G.R. Schmidt: Could it be possible that the same conditions necessary for a fine protein matrix are also necessary to form a pore free coating on fat droplets and that this coating actually entraps the lipid and prevents it from coalescing and assuming a non spherical shape? I notice that spheres are only fully coated when the entire protein gel is a fine laced aggregate.
Authors: It is generally true that the proteins involved in matrix formation also function in forming the IPF. It seems that conditions which favour the formation of a fine protein matrix will also result in an increased number of pore-free globules being formed. These conditions may include adequate extraction of myosin and actomyosin (Gordon and Barbut, in preparation). However, even batters with fine structured matrices had several fat globules with pores in their protein coats present (see Figs. 2 and 3) as a result of differences in the thickness and type of coat. Therefore, the actual occurrence of pores in the IPF may not be affected by the above-mentioned conditions since they appear to be present in each batter. However, the number of pore-bearing globules, the number of pores per globule, the size of the pores and whether or not they become rupture holes all appears to be affected by the same conditions which influence matrix structure.

We agree that the coating entraps the fat and helps to prevent it from coalescing and becoming non-spherical. The protein coat also serves as an intermediary to lipid-matrix binding as shown in this study (Figs. 3, 13 and 14) and in a previous study (Gordon and Barbut, 1990). This further helps to prevent coalescence.

C.M. Lee: You stated "it is difficult to see how fat not localized within a membrane could be stabilized by this entrapment mechanism during cooking". How do you justify this statement? Authors should be aware that entrapment follows localization, the formation of IPF is a result of protein-lipid interaction in which protein molecule orient to stabilize its molecular structure upon interaction with fat molecule. This can occur in the small surface area as in the case where fat is finely dispersed in relatively uniform size without disrupting matrix continuity. However, in either case when fat is not uniformly dispersed (e.g. low melting point fat or high chopping temperature) or when a noncohesive matrix is prepared with meat of poor functionality, no continuous and cohesive matrix is available to entrap the dispersed fat. One good example why the matrix continuity should be a prerequisite to the stable fat dispersion, is making a meat emulsion with squid protein which has a high level of salt soluble actomyosin to form IPF sufficient to cover the fat globules dispersed, but failed to form a stable meat batter (Saffle, 1973). It is mainly because squid protein is not capable of forming a continuous, rigid matrix which can entrap the fat particles and keep them from coalescing.
Authors: We have suggested that localization precedes entrapment, as you have pointed out. The cohesiveness and continuity of the matrix is undoubtedly of importance and its importance to fat stabilization was not questioned. We merely draw attention to the fact that mobile, liquid fat would move relatively freely through the openings in the protein matrix during cooking if it was not in some way localized within the system and kept away from other fat particles (see Fig. 6).

J.M. Regenstein: How does one determine that a droplet has lost fat and is still round?
Authors: The pores in the protein film are sites of fat loss from the globules (Fig. 4). It is therefore assumed that globules which show several pores by SEM have undergone at least limited fat exudation (Jones and Mandigo, 1982; Barbut, 1988). These globules still appear to retain their oval or spherical shape (Figs. 1 and 2). In addition, both TEM (Fig. 5) and SEM (Fig. 8) showed that globules in the process of exudation still kept their basic shape except for the section of the protein film surrounding the exuding fat.

F.W. Comer: Figure 1 shows an interesting collection of globules, two of which are identified as having thick rough coats. The number of holes is amazing. What is left inside? Has the fat exuded out and left empty "egg shells"? Other micrographs suggest a "budding" effect from a single pore. If fat were to exudate from a membrane-coated globule, I would expect some type of collapsed structure. Why is this not observed?
Authors: The globules shown in Figure 1 represent extreme cases of pore formation in thickly coated fat globules in an unstable treatment. In these extremely porous globules it is likely that some of the fat has been lost. However, because of the uniformity of distribution of the pores around the globules, extensive fat loss as shown in Fig. 4 probably did not occur and instead several small

amounts of fat probably exuded through each pore (see comparable situation in Fig. 5a), leaving a fair amount of the fat still inside the globules. Collapse of these structures was not seen possibly because the thick protein film denatured during cooking to form a rigid "shell" while the fat was still expanding (and exuding). Because the coat is protein, primary fixation with the aldehydes would further stabilize this rigid structure and prevent collapse during further processing in EM preparation. In addition, the protein coat is also bound to the rigid protein matrix which could help in retaining the shape despite some fat loss. However, if extensive, rapid fat loss occurs, before the protein coat has "set" during cooking then some collapse of the structure would be expected.

J.M. Regenstein: The fact that the procedure led to exudate in all cases would suggest that the conditions may not be realistic for real meat systems.
Authors: A certain degree of fat exudation was observed in all treatments with sufficient frequency to merit discussion as to its possible role in fat stabilization. However, it should be noted that not all fat globules showed exudation and that the phenomenon appeared to be related to the uniformity and thickness of the IPF around fat globules. Globules with very uniform protein coats (thick or thin) quite often did not show fat exudation. Thickly-coated, uniform globules are probably able to stabilize fat by virtue of their mechanical strength of their IPF while thinly coated, uniform globules would rely more on the elasticity of their IPF to retain fat and prevent fat loss.

A.M. Hermansson: An alternative to the proposed exudation mechanism, illustrated in Figure 8, could be flocculation of smaller droplets onto bigger globules and partial coalescence of the semicrystalline fat during cooling or during the preparation process for EM. Partial coalescence will not necessarily result in a lack of the spherical shape.
Authors: The main fat globules in Figure 8 show continuity of their protein coats with that of the smaller globules. This suggests that the protein coat around the "parent" and "daughter" globules is the same. The long connecting "neck" of protein in Figure 8a would not be seen in the case of coalescence. The unity of the protein coat around parent and exuded globules is seen quite clearly by TEM ("E", Fig 5b). In the case of flocculation, each globule would have its own distinct protein coat. With coalescence, protein residues would be seen within the fat (Fig. 4, unstable globule) as has been shown in other studies (Comer and Allan-Wojtas, 1988; Gordon and Barbut, 1989). Hence, while it is perhaps difficult to be definitive about fat exudation from SEM micrographs, TEM makes the distinction between exudation and coalescence clear.

G.R. Schmidt: In the TEM pictures, openings in the membrane are occasionally seen, but the pores seen in the SEM photos appear very deep. Why don't we see these deep pores in the TEM pictures?

Authors: This may be because the depth of the pores (as seen by SEM) is dependent on a) the thickness of the IPF and b) the internal organization of the fat globule. If the fat globule has an internal protein structure which is connected to the protein coat at the point of pore formation, then the pore will appear to extend deeply into the globule because it is merely an extension of the internal protein.

A.M. Hermansson: The multilayered structure shown in Figure 10 is interesting and focuses the attention to the potential of membrane residues of comminuted meat systems as stabilizers. Have the authors any information about variations in the multilayered structures?
Authors: In a follow-up study on the role of the IPF in raw meat batters, it was found that the multilayered structure varied between treatments and sometimes within the same meat batter (unpublished). The structure of the IPF appears to vary from a single layer to up to four different layers. We have found that several different soluble proteins are involved in IPF formation and that insoluble proteins may also form an IPF around fat globules (Gordon and Barbut, 1990 in preparation). We therefore believe that the structure of the protein coat varies depending on the types of proteins of which it is composed.

A.M. Hermansson: The statement of the domain shown in Figure 11 of being a large fat globule is not convincing. This could be a partly broken down fat cell aggregate where the network is residues of the collagen rich cell walls or some other structural component in the complex meat system. Please comment.
Authors: Your analysis of Fig. 11 could be correct. However, the size of each of these compartments (<1μm in diameter) makes it unlikely, in our opinion, that they are remnants of cell wall structures. In addition, such internal structures have been observed in several other large globules in this study (not shown) and in several of our other studies.

F.W. Comer: The presence of protein material inside fat globules has been observed before, but Figure 11 is possibly the best published example. You have attributed this to "protein which coats internal fat". I believe that this may be due to coalescence of protein coated fat globules (or alternatively, residual fat cell membranes which may be less likely for MDCM). It has always surprised me, that if protein coatings are prevalent in comminuted meat products, why are they not observed in coalesced fat pools? Protein should be pressed against protein in a restricted mobility environment, and some (partially) regular pattern observed. What do you believe happens to the protein coats when fat globules coalesce in a gelled meat system?
Authors: Residual protein coats within pools of coalesced fat are occasionally seen (Fig 4) and were highlighted in our previous study (see Fig. 2c, Gordon and Barbut, 1989). In fact, in some of your work (Comer and Allan-Wojtas, 1988) you presented micrographs which we believe show a similar situation. However, this occurrence is not

very common in unstable batters. We believe that this is because instability results either from the rupture of the protein coat (thereby releasing fat) during cooking or its absence around much of the fat prior to cooking as has been shown in another study (Gordon and Barbut, submitted). It would therefore be the free fat itself in most cases which coalesces and not so much the fat particles/globules. The protein coats (if any) originally present around these globules would remain bound to the protein matrix and not be present (in most cases) within the coalesced pool of fat.

C.M. Lee: I would agree with the authors view on the role of the IPF as described in the paper. But, I must point out that the formation of an IPF is a mere consequence of physicochemical interactions bewteen the protein and lipid which determine the fate of the thermal stability of meat emulsions. They include protein functionality, the extent of protein solubilization, matrix integrity, fat dispersion pattern (particle size and density) and other factors that further alter the fat morphology, independent of the formation of an IPF.

Authors: As you have indicated, there are many factors which influence batter stability. We believe that there may be a greater interdependence among these factors than is generally acknowledged and that IPF formation is one of the more important factors which interacts with others to determine meat batter stability.

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