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INTERFACIAL INTERACTIONS, COMPETITIVE ADSORPTION AND EMULSION STABILITY

Jianshe Chen, Eric Dickinson and Graeme Iveson

Procter Department of Food Science
University of Leeds
Leeds LS2 9JT
England

Abstract

Food emulsion stability is strongly influenced by the interactions in the adsorbed layer around emulsion droplets. Competitive adsorption of pure milk proteins (β-casein or β-lactoglobulin) with nonionic surfactants in oil-in-water emulsions is shown to depend on the age of the adsorbed protein layer. Evidence is presented for slow accumulation of protein at the oil-water interface and/or partition of protein into the oil phase of emulsion droplets containing oil-soluble surfactant associated in reversed micelles. Small amounts of added water-soluble surfactant or calcium ions are shown to have a pronounced effect on the droplet coalescence rate under turbulent shear flow conditions. It appears that orthokinetic emulsion stability is related to the composition and surface rheology of the adsorbed layer and to the degree of perikinetic flocculation of the emulsion droplets.

Key Words: Protein layers, competitive adsorption, orthokinetic stability, surfactant, β-lactoglobulin, β-casein, surface rheology, shear-induced coalescence, calcium ions, flocculation

Introduction

Most food emulsions of the oil-in-water type are stabilized primarily by an adsorbed layer of protein forming a protective steric barrier around the dispersed droplets. The interfacial region typically also contains various surface-active lipid components which may have originated as impurities in the oil or fat phase or may have been added deliberately during formulation. Additional macromolecular stabilization may be provided by non-adsorbing polysaccharide acting as a thickening or structuring agent in the aqueous continuous phase, or by the adsorption of interacting polysaccharide molecules on top of the primary protein layer (Dickinson, 1992).

While the general relationship between interfacial interactions and the stability of emulsions is reasonably well established in formal terms (Dickinson and Stainsby, 1982; Tadros and Vincent, 1983; Walstra, 1993b), it is fair to say that our ability to make reliable quantitative predictions of stability for complex food systems is extremely limited (Walstra, 1988). This is in part because there has been, until very recently, a lack of even the most rudimentary information on how individual food components (protein, polysaccharide, lipid) are quantitatively distributed between the bulk phases and the interfacial region. This situation has improved a little over the past couple of years, insofar as we do now have interfacial composition data for a few simple mixed model systems containing pure milk proteins and small-molecule surfactants (Dickinson, 1991, 1992). Now is perhaps an appropriate time, therefore, to attempt to use these same simple model systems as the basis for a new systematic study of the effect of emulsion composition on stability with respect to the usual criteria of flocculation, creaming and coalescence (Dickinson, 1988). If we are seriously to address the relationship between product composition and product quality, we must also pay more attention than previously to the consequences of time-dependent
changes in adsorbed layer structure and composition, since in practice the assessment of stability is rarely carried out on a freshly made emulsion—but rather on one that has been stored for a period of hours, days or even longer.

This paper presents results of experiments which attempt to address some of these issues. Firstly, we describe the influence of emulsion ageing on the competitive adsorption of pure milk proteins and small-molecule surfactants. Secondly, we consider the shear-induced coalescence stability of (i) β-lactoglobulin emulsions containing various amounts of water-soluble emulsifier and (ii) casein emulsions containing various concentrations of calcium ions. These stability studies were motivated by the commercial importance of shear-induced aggregation and coalescence in the processing of dairy emulsions and by the known sensitivity of the stability of these systems to small quantities of surfactants or calcium salts.

**Materials and Methods**

The proteins β-casein (2.40×10^4 daltons) and β-lactoglobulin (1.84×10^4 daltons) were obtained from Sigma Chemicals, as was the commercial-grade emulsifier Tween 20 (polyoxyethylene(20) sorbitan monolaurate, 1.23×10^3 daltons). The research-grade surfactants, C_{12}E_{2} (diethylene glycol n-dodecyl ether, 2.74×10^2 daltons) and C_{12}E_{8} (octaethylene glycol n-dodecyl ether, 5.38×10^2 daltons), were obtained from Fluka Chemicals. Hydrocarbon oils and buffer salts were AnalaR-grade reagents. Oil-in-water emulsions were prepared by high-pressure homogenization. The lipophilic surfactant C_{12}E_{2} was dissolved in the hydrocarbon oil phase prior to emulsification; the hydrophilic surfactant (C_{12}E_{8} or Tween 20) was added following emulsification after the emulsion had been allowed to age for a fixed length of time. A Malvern Mastersizer was used to determine the droplet-size distribution, the volume–surface average diameter d_{32}, and the total interfacial area per unit mass of emulsion. Protein concentrations in the serum after centrifugation were determined using a Pharmacia fast protein liquid chromatography system. In the orthokinetic stability experiments, emulsions were sheared at a constant speed of ~10^4 r.p.m. using a Silverson-type blender (power input up to 1 MW m⁻³), and samples were taken every 15 minutes for droplet-size analysis. Detailed information on materials and methods may be found elsewhere (Chen and Dickinson, 1993; Dickinson et al., 1993).

**Time-Dependent Aspects of Competitive Adsorption**

A milk protein like β-casein or β-lactoglobulin saturates the oil–water interface at a much lower bulk concentration than a water-soluble non-polymeric emulsifier such as C_{12}E_{8} or Tween 20. This greater thermodynamic affinity of the protein for the adsorbed state is consistent with a greater lowering of the equilibrium interfacial tension by the protein than by the small-molecule surfactant at the same low (molar) bulk concentration. A converse situation applies at high bulk concentrations, however, where the surfactant produces a lower equilibrium tension and a more efficiently packed dense monolayer. This is probably the main thermodynamic basis for the competitive displacement of milk protein from the oil–water interface in food colloids by various emulsifiers of different hydrophilic–lipophilic balance. Milk protein displacement by monoglycerides has been observed directly using confocal scanning laser microscopy (Heertje et al., 1990). Using the indirect depletion method applied to model emulsions stabilized by either β-casein or β-lactoglobulin, it has been shown by us (Courthaudon et al., 1991a, 1991b, 1991c) that the addition of water-soluble nonionic surfactant (C_{12}E_{8} or Tween 20) to a fresh oil-in-water emulsion leads to complete displacement of protein from the interface if the surfactant/protein molar ratio is high enough (R > 20). It has also been demonstrated (Dickinson and Tanai, 1992) that the quantity of water-soluble surfactant required for complete displacement is affected by the presence of oil-soluble surfactant (C_{12}E_{2} or pure monoglyceride) in the dispersed phase (liquid n-alkane or purified soybean oil). In all of these previous depletion studies, the water-soluble surfactant was either present during homogenization or added immediately afterwards (within a few minutes). As it is well known that protein layers change with time, it is reasonable to ask whether the ageing of the emulsion has any appreciable effect on the inferred competitive adsorption behaviour.

Protein surface coverage data are shown as a function of time in Figure 1 for emulsions stabilized by β-casein (0.4 wt% protein, 20 wt% hydrocarbon oil, pH 7). Surface concentrations were inferred from the bulk protein concentration measured in the aqueous phase after emulsion centrifugation (Chen and Dickinson, 1993). The emulsion made with pure n-hexadecane as the oil phase has a protein surface coverage of Γ = 2.95 ± 0.05 mg m⁻² and almost all the protein present in the sample (93%) is adsorbed at the oil–water interface. In the emulsion containing 0.1 wt% C_{12}E_{2} (i.e. 0.5 wt% C_{12}E_{2} dissolved in the n-hexadecane), the surface...
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coverage is reduced to $\Gamma = 2.30 \pm 0.05$ mg m$^{-2}$. This lower coverage is not due to any significant loss of $\beta$-casein to the aqueous phase—there is still some 90% adsorbed—but simply to the smaller average droplet size $d_{32}$ (larger total surface area) created in the presence of the lipophilic surfactant (Courthaudon et al., 1991a). The surface concentration in both these samples does not change with the time $t$ of storage after emulsion formation. Shown also in Figure 1 are results for a pure n-hexadecane-in-water emulsion to which Tween 20 at a surfactant/protein molar ratio $R$ was added at various times following emulsion preparation. Again we see that the inferred surface coverage ($\Gamma = 2.60 \pm 0.05$ mg m$^{-2}$) does not change with time over the 3–4 hour storage period.

We now turn to emulsions made with the globular protein $\beta$-lactoglobulin, which gives similar droplet sizes to $\beta$-casein but with only about one half of the protein present adsorbed at the fresh oil–water interface. Figure 2 shows the effect of oil-soluble surfactant on the time-dependent surface concentration in emulsions containing 0.4 wt% $\beta$-lactoglobulin and 20 wt% n-hexadecane. We see that, whereas the surface coverage remains constant at $\Gamma \approx 1.4$ mg m$^{-2}$ in the absence of $C_{12}E_2$, there is a steady increase in $\Gamma$ up to nearly 1.8 mg m$^{-2}$ after 5 hours when 0.1 wt% $C_{12}E_2$ is present. (This concentration of $C_{12}E_2$ corresponds to a lipophilic surfactant/protein molar ratio of 17:1.) Time-dependent effects are also observed in emulsions to which Tween 20 is added after various ageing times $t$ (see Figure 3). The Tween 20 is added at a level (surfactant/protein molar ratio $R = 15$) which just gives complete displacement of $\beta$-lactoglobulin from the surface of freshly made emulsion droplets without $C_{12}E_2$. We see that, within experimental error ($\pm 0.05$ mg m$^{-2}$), $\Gamma(t)$ increases roughly linearly with time $t$ over the first 4 or 5 hours irrespective of whether $C_{12}E_2$ is present or not. The values of $\Gamma$ are, nevertheless, substantially higher in the system containing $C_{12}E_2$: after 24 hours the (steady-state) value is $\Gamma(24) = 1.38$ mg m$^{-2}$ as compared with $\Gamma(24) = 0.65$ mg m$^{-2}$ in the system without $C_{12}E_2$.

**Figure 1.** Time dependence of surface concentration $\Gamma$ of $\beta$-casein in oil-in-water emulsions (0.4 wt% protein, 20 wt% n-hexadecane, $d_{32} = 1.17$ µm, pH 7, 20 °C): o, no surfactant present; $\Delta$, Tween 20 ($R = 5$) added after various storage times $t$; o, 0.1 wt% $C_{12}E_2$ present before homogenization ($d_{32} = 0.91$ µm), $\Gamma$ measured after various storage times $t$.

**Figure 2.** Time dependence of calculated surface coverage of $\beta$-lactoglobulin in oil-in-water emulsions (0.4 wt% protein, 20 wt% n-hexadecane, pH 7, 20 °C). Apparent protein surface concentration $\Gamma$ is plotted against the storage time $t$: o, no surfactant present; $\Delta$, 0.1 wt% $C_{12}E_2$ present before homogenization ($C_{12}E_2$/$\beta$-lactoglobulin molar ratio = 17).

**Figure 3.** Influence of the age of the protein film on displacement of $\beta$-lactoglobulin by Tween 20 ($R = 15$, added after emulsification) from the oil–water interface in oil-in-water emulsions (0.4 wt% protein, 20 wt% n-hexadecane, pH 7, 20 °C). Apparent protein surface concentration $\Gamma$ is plotted against the storage time $t$ prior to the addition of water-soluble surfactant: o, no $C_{12}E_2$ present; $\Delta$, 0.1 wt% $C_{12}E_2$. 

A comparison of competitive displacement curves for β-lactoglobulin + Tween 20 in freshly prepared emulsions (pH 7, 20 °C) with those in emulsions left for 3 hours prior to addition of Tween 20 clearly shows (Chen and Dickinson, 1993) that a larger amount of water-soluble surfactant is required for the complete displacement of the β-lactoglobulin from the aged oil–water interface. This finding seems to be consistent with an earlier study (de Feijter et al., 1987) on competitive adsorption in concentrated emulsions in which the surface coverage was reported as \( \Gamma > 0.6 \text{ mg m}^{-2} \) for Tween 20 \((R \approx 15)\) added some two hours after emulsion formation.

There are two possible mechanisms for the increase in apparent protein surface coverage with time in the β-lactoglobulin emulsions with \( C_{12}E_2 \) dissolved in the oil phase. One mechanism involves the protein becoming slowly incorporated into an oil phase containing excess lipophilic surfactant organized in the form of an aggregated solution, i.e. reversed micelles. (In this case the quoted \( \Gamma \) value is not the real adsorption but just an apparent adsorption.) That such an incorporation is feasible in principle is supported by the widely reported activity of enzymes in reversed micellar solutions (Martinek, 1989). On the other hand, a high surfactant/protein ratio is usually necessary for substantial solubilization of protein into a reversed micellar phase. In our systems the ratio of oil-soluble surfactant to protein is relatively low (1:4 by weight) and there is a large surplus of water; both of these factors would normally tend to reduce the amount of protein incorporation into the oil phase.

In the second mechanism, the presence of adsorbed \( C_{12}E_2 \) would allow more β-lactoglobulin to accumulate at the emulsion droplet surface, perhaps due to the greater macromolecular mobility and ease of protein unfolding at the interface. One might further argue that, in the presence of oil-soluble surfactant, more space becomes available for additional β-lactoglobulin adsorption into the “gaps” in the developing primary layer structure (i.e. it gradually becomes a little more like the disordered protein β-casein); alternatively, there could be linking of native protein molecules to existing partially denatured adsorbed molecules to form some sort of secondary layer. A possible argument against this interpretation, however, is that the presence of \( C_{12}E_2 \) at the interface should reduce the effective hydrophobicity of the oil phase, thereby reducing the thermodynamic driving force for surface denaturation. Protein coverages at the polar triglyceride–water interface are generally lower than those at the non-polar hydrocarbon–water interface (Dickinson and Tanai, 1992).

Each of the above mechanisms would lead to an increasing depletion of β-lactoglobulin from the aqueous phase and hence to an inferred increase in protein surface coverage with time. There seems no reason, at least in principle, why both mechanisms should not occur simultaneously at different rates. An argument in favour of some solubilization of β-lactoglobulin into a reversed micellar dispersed phase is that, when excess Tween 20 is added to a 3-hour-old emulsion containing 0.1 wt% \( C_{12}E_2 \), a significant amount of the protein (i.e. equivalent to 0.25 mg m\(^{-2}\)) remains undisplaced (undisplaceable) from the emulsion droplets at surfactant/protein molar ratios up to \( R \approx 100 \). This quantity of β-lactoglobulin is similar to that which becomes associated with the droplets after 3 hours in the absence of Tween 20 (see Figure 2). It is pertinent to note the difference in behaviour of emulsions of β-lactoglobulin (Figure 2) and β-casein (Figure 1) prepared under identical emulsification conditions. The reason why β-casein does not partition into the putative reversed-micellar dispersed oil phase is that almost all of the protein present is adsorbed at the oil–water interface (Courthaudon et al., 1991a). As there is no significant reservoir of available protein in the continuous phase of the β-casein emulsion, it is clearly impossible for the surface coverage to show any increase with time without loss of total interfacial area.

In β-lactoglobulin emulsions without \( C_{12}E_2 \), where reversed micelles can presumably be discounted, the increase in \( \Gamma \) with \( t \) on adding Tween 20 after various storage times \( t \) (see Figure 3) must be due to a genuine ageing effect in the adsorbed layer which makes some of the globular protein molecules less easy to displace. We know already that there is a slow cross-linking of whey protein molecules at the emulsion droplet surface via sulphhydril–disulphide interchange (Dickinson and Matsumura, 1991) and that the rate of cross-linking is rather insensitive to the presence of Tween 20 (Courthaudon et al., 1991c). It may be that displacement of protein oligomers from an aged film containing a greater proportion of unfolded (denatured) molecules is more difficult than displacement of quasi-native β-lactoglobulin molecules from the surface of fresh emulsion droplets.

Coalescence of Emulsions in Shear Flow

Effect of Surfactants

The liquid droplets in an emulsion may break apart or join together under the influence of laminar or turbulent shear flow. Whether droplet disruption predominates over droplet coalescence, or vice versa,
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depends on various factors—the average droplet size, the type and strength of the fluid flow, the chemical nature of the emulsifier, and so on (Walstra, 1993a).

When the dispersed particles are solid, shear forces may lead to changes in the formation, rearrangement and disruption of aggregates, with average aggregate structure being dependent on the flow conditions (Oles, 1992). The susceptibility of an initially stable emulsion (or particulate dispersion) to coalescence (or aggregation) in a flow field is known as orthokinetic stability. This type of stability depends *inter alia* on a balance between interdroplet forces and hydrodynamic forces, and on the susceptibility to rupture of the thin liquid film between colliding droplets (Tadros and Vincent, 1983; Lips *et al*., 1993). This balance, in turn, depends on the composition and structure of the adsorbed layer at the oil–water interface. Systems containing both protein and surfactant are of particular food science interest since it is well established (Goff and Jordan, 1989; Barfod *et al*., 1991) that the addition of emulsifiers (oil- or water-soluble) enhances the shear-induced clumping of the fat globules in dairy emulsions such as whipping cream and ice-cream. The mechanisms underlying the role of fat crystals in the partial coalescence of semi-solid droplets have recently been discussed by Boode and Walstra (1993). To avoid complications from fat crystals here, our emulsions are composed of fully liquid droplets.

In model oil-in-water emulsions (0.45 wt% β-lactoglobulin, 10 wt% hydrocarbon oil) sheared continuously under turbulent flow conditions, we have observed (Dickinson *et al*., 1993) that a small amount of Tween 20 can have a substantial influence on the orthokinetic stability. Figure 4 shows plots of average (volume/surface) droplet diameter $d_{32}$ against shearing time for emulsions with surfactant/protein molar ratio in the range $0 \leq R \leq 1$. With no Tween 20 added after emulsification, the control protein-stabilized emulsion (pH 7) shows no systematic change in average droplet size ($d_{32} = 0.36 \pm 0.05 \mu m$) over a period of ~3 hours. Thereafter, however, the mean droplet diameter begins to increase at a gradually more rapid rate, reaching a maximum value of $d_{32} \approx 4.5 \mu m$ at 300 minutes, and then falling back slightly at longer times. In the pure protein emulsion, the characteristic destabilization time corresponding to the maximum value of $d(d_{32})/dt$ is estimated to be $t_d = 290 \pm 10$ minutes. With Tween 20 added to the fresh protein-stabilized emulsion within 10 minutes of homogenization, the value of $t_d$ is shifted to lower values. Figure 5 shows a plot of destabilization time $t_d$ versus molar ratio $R$. We can see that the destabilization rate is about an order of magnitude faster at $R \approx 1$ than at $R = 0$.

The average droplet diameter remains roughly constant at long times ($t \rightarrow \infty$) because the rate of coalescence is equal to the rate of droplet disruption under steady state conditions. In the early stages of shearing, the droplet size also remains roughly constant because the coalescence rate of the small droplets in the freshly made emulsions is very low and the droplet disruption rate is even lower. But, after an apparent lag

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\[ \text{Figure 4. Influence of emulsifier Tween 20 added after homogenization on the shear-induced coalescence of emulsion droplets (0.45 wt% β-lactoglobulin, 10 wt% n-tetradecane, pH 7). The average diameter $d_{32}$ is plotted against the shearing time $t$ for various surfactant/protein molar ratios: (a) $R = 0$; (b) $R = 0.125$; (c) $R = 0.25$; (d) $R = 0.5$; (e) $R = 1.0$.} \]

\[ \text{Figure 5. Plot of destabilization time } t_d, \text{ defined as the time when } \frac{d(d_{32})}{dt} \text{ reaches a maximum, as a function of surfactant/protein molar ratio } R \text{ in emulsions containing β-lactoglobulin + Tween 20.} \]
phase, the plot of $d_{32}$ versus $t$ for $R = 0$ shows a strong
divergence in the coalescence rate. Qualitatively similar
behaviour was also reported recently by Lips et al.
(1993). This pronounced divergence arises because the
second-order Smoluchowski rate constant for the
coalescence of two droplets of diameters $d_i$ and $d_j$ is
proportional to $(d_i + d_j)^3$ (Dickinson et al., 1993). The probability of coalescence for a pair of colliding
droplets of diameter 0.4 μm is therefore $10^3$ times
smaller than for a pair of diameter 4 μm. The relative
change in average droplet size caused by shearing is
more or less the same in the presence of surfactant, but
the absolute value of the coalescence rate constant (and
hence of the inverse destabilization time $1/t_d$) is
substantially increased due to the influence of surfactant on the adsorbed protein layer.

![Figure 6](image6.png)

**Figure 6.** Influence of the initial average droplet
diameter $d_{32}(t=0)$ on shear-induced coalescence of
emulsion droplets (0.4 wt% β-lactoglobulin, 20 wt% n-
tetradecane, pH 7). The average diameter $d_{32}$ is plotted
against shearing time $t$: o, $d_{32}(t=0) = 0.35$ μm; •,
$d_{32}(t=0) = 0.48$ μm; Δ, $d_{32}(t=0) = 0.68$ μm.

The results in Figure 6 illustrate the effects of oil
content and initial droplet size $d_{32}(t=0)$ on the plot of
$d_{32}$ versus shearing time $t$. The data refer to pure β-
lactoglobulin emulsions (0.4 wt% protein, 20 wt% n-
tetradecane, pH 7) with three initial mean droplet
diameters: $d_{32}(t=0) = 0.35$ μm (based on average of 3
separate experiments), $d_{32}(t=0) = 0.48$ μm (from 2
experiments), and $d_{32}(t=0) = 0.68$ μm (2 experiments).
The different values of $d_{32}(t=0)$ were obtained by
adjusting the homogenization conditions. The aqueous
phase protein concentration (0.5 wt%) in these
emulsions is the same as that for those in Figures 4 and
5, but the overall oil/protein ratio is more than twice as
large. From the data in Figure 6, we see that the
eмуsion with $d_{32}(t=0) = 0.35$ μm has a smaller
destabilization time ($t_d \approx 85$ minutes) than the value
reported earlier ($t_d \approx 290$ minutes) for the equivalent
emulsion of lower volume fraction (i.e. see curve (a) in
Figure 4). A reduction in destabilization time with
increasing dispersed phase volume fraction has been
noted previously by Lips et al. (1993). We also note
from Figure 6 that, at constant oil volume fraction, the
destabilization time $t_d$ decreases substantially with
increasing initial mean droplet diameter $d_{32}(t=0)$. This
is consistent with the interpretation of the divergence in
terms of a coalescence rate constant whose magnitude
is a strongly increasing function of droplet size. The
fitted exponent $m = 1.2 \pm 0.1$ in $t_d \sim [d_{32}(t=0)]^m$ is
rather lower than predicted by simple Smoluchowski
theory. This may partially be due to the fact that the
emulsion with lower $d_{32}(t=0)$ has droplets with a lower
protein surface coverage and hence poorer steric
protection against coalescence. In addition, it could be
that $d_{32}$ is not the most appropriate droplet size
parameter to include in the scaling relationship (Lips et
al., 1993).

![Figure 7](image7.png)

**Figure 7.** Competitive adsorption and surface shear
toughology of β-lactoglobulin + Tween 20 at the
oil–water interface. Protein surface concentration $\Gamma$ (A)
is plotted against the molar ratio $R$ in oil-in-water
eemulsions (0.45 wt% protein, 10 wt% n-tetradecane,
pH 7). Apparent surface shear viscosity $\eta$ (O) after 5
hours adsorption from a $10^{-3}$ wt% aqueous protein
solution (pH 7, 25 °C) is plotted against the molar
ratio.

The reason why such small amounts of surfactant
produce such large effects on the orthokinetic stability
can perhaps be better understood by considering the
complementary data set out in Figure 7. Though the
protein is completely displaced from the fresh emulsion
droplet surface by Tween 20 added at molar ratio $R \geq
10$, there is very little displacement at $R \leq 1$. It would
seem possible, therefore, that orthokinetic instability is
triggered initially, not simply by extensive protein
displacement, but by some change in adsorbed layer
properties caused by incorporation of surfactant into the adsorbed protein film. One kind of adsorbed film property which can change dramatically in the presence of very small amounts of surfactant is the surface shear viscosity. This is also illustrated in Figure 6 for the case of mixed β-lactoglobulin + Tween 20 at the planar hydrocarbon oil–water interface (Courtaudon et al., 1991b). The drop in surface shear viscosity of adsorbed β-lactoglobulin at pH 7 for molar ratios over the range 0 ≤ R ≤ 1 correlates quite strongly with the loss of orthokinetic stability when Tween 20 is added to a β-lactoglobulin emulsion at the same low relative concentrations. This implies that the influence of a very low concentration of water-soluble surfactant in increasing the rate of shear-induced droplet coalescence is explicable in terms of a loosening of the adsorbed protein film as reflected in the greatly reduced surface shear viscosity. Some competitive protein displacement undoubtedly occurs, and increasingly so as R increases, but it seems that the displacement per se (at least under quiescent conditions) may not be the prerequisite for the onset of orthokinetic destabilization in the presence of very small amounts of added surfactant.

Additional support for a correlation between orthokinetic stability and surface rheology is provided by experiments on systems containing a mixture of β-lactoglobulin with an oil-soluble surfactant. In contrast to the large reduction found with Tween 20, the presence of Span 80 (sorbitan monooleate) in the hydrocarbon oil phase at molar ratios of up to R = 32 leads to only a modest reduction (10–30%) in the estimated destabilization time \( t_d \) (A. Williams, unpublished results). Also, under complementary conditions to those applicable to the data in Figure 7, it has been separately found (J. Chen, unpublished results) that the presence of lipophilic surfactant (C_{12}E_2) in the oil phase has relatively little effect on the measured surface shear viscosity—in contrast to the water-soluble surfactant which gives a large reduction. These combined results suggest that there may be a link between (i) structure and viscoelasticity of the adsorbed protein layer under conditions of very mild deformation in the surface rheometer and (ii) coalescence stability of these emulsions in vigorous turbulent flow. What we do not know, however, is how the high shear-rates present during the orthokinetic stability experiments affect the distribution of species between bulk and interface. (The competitive adsorption experiments are carried under conditions of mild stirring and surface shear viscometry is performed at extremely low shear-rates.) Possibly of more direct relevance to the orthokinetic stability are dynamic dilational surface rheological properties like those reported recently by Clark et al. (1993).

**Effect of Calcium Ions**

Any factor that affects the degree of flocculation of emulsion droplets might also be expected to affect other properties such as orthokinetic coalescence stability (Dickinson and Stainsby, 1982). For the case of emulsions prepared with casein as the primary emulsifier, an important factor influencing perikinetic flocculation is the concentration of calcium ions present before or after homogenization (Hunt et al., 1993). The onset of flocculation may be defined as the calcium ion concentration above which there is an apparent increase in some average droplet diameter (e.g. \( d_{32} \)) as measured by the Mastersizer. According to this criterion, an oil-in-water emulsion (1 wt% sodium caseinate, 20 wt% hydrocarbon oil, pH 7) is said to become flocculated when it contains 8 mM calcium ions or above. This flocculation can be reversed by dilution (Dickinson et al., 1992), and also to a large extent by vigorous stirring, so that a range of emulsions of average droplet size \( d_{32} = 0.43 \pm 0.03 \mu m \) can be prepared with calcium ion concentrations in the range 0–14 mM which can then form the starting emulsions \( t = 0 \) for the shearing experiments whose results are presented in Figure 8. The plots of \( d_{32} \) versus shearing time in were produced under similar flow conditions (9000 rpm in a MSE Silverson blender) to those for the emulsions containing Tween 20 (Figure 4). And, at the higher calcium ion concentrations, the plots exhibit the same general qualitative form: a lag phase, where \( d_{32} \) remains close to that of the initial emulsion, followed by a rather sudden jump to an average steady-state droplet size of magnitude some 15–20 times larger.

**Figure 8.** Influence of calcium ions added after homogenization on the shear-induced coalescence of emulsion droplets (1 wt% sodium caseinate, 20 wt% n-tetradecane, pH 7). The average diameter \( d_{32} \) is plotted against the shearing time \( t \). The numbers on the curves denote the concentrations of added calcium ions in the emulsions (in units of millimoles per litre).
The calcium-free caseinate emulsion (labelled "0") in Figure 8 is approximately as stable as the pure $\beta$-lactoglobulin emulsion ($R = 0$) in Figure 4, and the plot of $d_{32}$ versus shearing time remains exactly the same up to 6 mM calcium chloride concentration. In the presence of 8 mM CaCl$_2$, we observe a slight loss of orthokinetic stability as $d_{32}$ versus $t$ begins to deviate significantly from the calcium-free case after $t = 120$ minutes (reaching $d_{32} \approx 2 \mu$m after 3 hours). However, just increasing the Ca$^{2+}$ content from 8 mM to 10 mM leads to a dramatic increase in the coalescence rate, so that the destabilization time in the casein emulsion reduces the stability further, but the relative magnitude of the change is less dramatic.

It does seem noteworthy that the calcium ion concentration range which induces the largest change in shear-induced coalescence stability (i.e. 8–10 mM CaCl$_2$) is identical to that which induces flocculation under static quiescent conditions. Even though all except the very smallest flocs are broken up by the severe hydrodynamic forces—$d_{32}$ remains low for $t \leq 15$ minutes—it is clear that the presence of a significant attractive interaction between the droplets [caused by the calcium ions (Hunt et al., 1993)] produces an order of magnitude increase in the effective coalescence rate as compared with the calcium-free emulsion. The implication would seem to be that a factor which increases the degree of flocculation under quiescent conditions, even when that flocculation is readily reversible, is also likely to reduce emulsion stability in shear flow. It would be interesting to test this hypothesis further by examining orthokinetic stability as a function of other variables such as temperature, pH or ionic strength.

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Discussion with Reviewers

B. Bergenståhl: The shear-induced destabilization of the emulsions occurs after a significant lag time (up to 3 hours). What types of processes are going on in the emulsion during this time? The particle size displays a slight decrease.

Authors: One process which is undoubtedly occurring to some extent is incorporation of air into the emulsion. The presence of air bubbles can be detected when an emulsion sample is immediately placed in the light-scattering cell of the Mastersizer, but the contribution from the bubbles can readily be made to disappear by sonication. It is known that air incorporation greatly enhances the clumping of partially crystalline fat globules during the whipping of cream, and so it would not be too surprising if it also had some effect on the kinetics of liquid droplet coalescence. Our experiments were carried out in a conventional high-speed laboratory blender; so some air incorporation is inevitable. But we certainly do not produce anything resembling whipped cream in our experiments—the emulsion is still a low viscosity liquid and we see no evidence of oil spreading at the air-water interface (i.e. no free oil is released). Nevertheless, in order to investigate the possible role of air bubble incorporation on the coalescence process, we are designing a closed shear cell for use in future experiments.

Another process occurring during the lag phase is ageing of the adsorbed protein film. This ageing will affect the mechanical properties of the droplet surface and the strength of the interdroplet interactions. Intuitively one might expect that an aged film would exhibit improved coalescence stability due to stronger intermolecular interactions within the adsorbed layer, but there may also be a greater susceptibility towards flocculation due to the formation of intermolecular cross-links between droplets. Intense flow conditions might be expected to enhance the rates of these processes by greatly increasing the number of protein–surface and surface–surface encounters. Yet another complication is the possible change in adsorbed layer structure and composition due to the applied flow.

The slight reduction in mean droplet size during the lag phase of curve (a) in Figure 4 may not be very significant. It could be associated with some inefficient sampling during a long experiment—there is a steady decrease in total emulsion volume as samples are removed. When samples are taken less frequently, the effect seems largely to disappear.

B. Bergenståhl: An interesting observation is that instability towards shear-induced coalescence appears
already at a surfactant/protein ratio less than \( R = 1 \), which is much before protein is displaced from the interface. Is the interfacial tension changed at this concentration? If so, could the change in tension be an explanation instead of change in interfacial viscosity?

**Authors:** Tension measurements at the same oil–water interface are unfortunately not available for the \( \beta \)-lactoglobulin + Tween 20 system. It would certainly be worthwhile to obtain such data to test your hypothesis. Tension measurements reported for this system at the air–water interface [Coke M, Wilde P J, Russell E J, Clark D C (1990) J. Colloid Interface Sci. 138, 489–504] indicate a slight lowering of the tension \( \gamma \) at low \( R \); a plateau region (little change in \( \gamma \)) in the range 0.2 < \( R < 2 \) was attributed by the authors to 1:1 surfactant–protein complexation. Surface tensions in the presence and absence of Tween 20 were found by Coke et al. (1990) to become equal at \( R \sim 5 \), i.e. at a surfactant concentration where much of the protein had presumably been displaced from the interface. In the same investigation, the workers also measured the protein mobility at the air–water interface as well as the foam stability. They concluded that the maintenance of viscoelasticity at the air–water interface is of paramount importance in determining foam stability in systems containing a mixture of protein + surfactant.

We can speculate on the likely importance of equilibrium interfacial tension effects at the \( n \)-tetradecane–water interface by referring to some earlier measurements of ours [Dickinson E, Euston S R, Woskett C M (1990) Prog. Colloid Polym. Sci. 82, 65–75] on sodium caseinate (0.1 wt%, pH 7) + C\( \text{12} \)E\( \text{8} \). We found that the presence of just \( 10^{-4} \) wt% surfactant (\( R < 0.1 \)) led to a big reduction in the surface shear viscosity but no significant change (\(< 0.5 \) mN m\(^{-1}\)) in interfacial tension. There is, of course, a difficulty in trying to compare directly the results from macroscopic surface experiments with those from emulsion experiments. This is because, for the same surfactant/protein ratio, the area of oil–water interface in the emulsion (per unit volume of aqueous phase) is typically 3 or 4 orders of magnitude larger than in the macroscopic surface experiment. What can be said without ambiguity, however, is that the amount of nonionic water-soluble surfactant (Tween 20 or C\( \text{12} \)E\( \text{8} \)) required for complete protein displacement is roughly that required to form a complete saturated surfactant monolayer at the interface. Conversely, surfactant significantly affects the orthokinetic stability of the same emulsions at total surfactant concentrations corresponding to just a few per cent of saturated monolayer coverage.

**W. Buchheim:** Real food emulsions often contain an oil (fat) phase consisting of a complex mixture of individual fat fractions resulting in specific melting and crystallization characteristics. Furthermore, emulsion formation often takes place at rather high temperatures (e.g. 60–80 °C) whereas the storage temperature may be 5–20 °C. It is important to know to what extent the interfacial interactions described in this paper are valid for fats which partly crystallize during storage.

**Authors:** The general thermodynamic aspects of surfactant/protein competitive adsorption should be the same for partly crystalline droplets as for wholly liquid droplets. It would not be surprising, however, to find some quantitative differences in the displacement curves due to differences in binding energies of proteins (and surfactants) at solid and liquid triglyceride surfaces. We know already [Dickinson E, Tanai S (1992) J. Agric. Food Chem. 40, 179–183] that protein coverages at liquid triglyceride–water interfaces are rather lower than at the corresponding liquid hydrocarbon–water interfaces. It would certainly be worthwhile also to carry out similar competitive adsorption measurements on emulsions containing solid fat particles. For our orthokinetic stability experiments, we deliberately chose a system of liquid droplets in order to avoid the complication of fat crystal bridging between droplets which is well known to increase very substantially the rate of globule clumping (partial coalescence) in flowing emulsions.

In addition to its influence on the fat phase, one perhaps should also consider the influence of temperature on the adsorbed layer. Over the low temperature range 0–20 °C, we have recently observed significant changes in the amount of \( \beta \)-casein adsorbed in liquid soya oil-in-water emulsions in the presence of oil-soluble and/or water-soluble surfactants [Dickinson E, Tanai S (1992) Food Hydrocolloids 6, 163–171]. It would be interesting to extend such measurements to, say, 70 or 80 °C, especially for systems containing \( \beta \)-lactoglobulin. Substantial compositional differences could possibly emerge at high temperatures due to known changes in protein structure and functionality associated with thermal denaturation.

**D. G. Dalgleish:** The authors demonstrate the importance of interfacial shear viscosity in determining the orthokinetic stability of Tween/\( \beta \)-lactoglobulin emulsions. However, it is then demonstrated that the stability of a caseinate emulsion is approximately the same as for a pure \( \beta \)-lactoglobulin emulsion. We know from previous work [Dickinson E, Rolfe S E, Dalgleish D G (1989) Int. J. Biol. Macromol. 12, 189–194] that the surface shear viscosities of these two proteins are
very different, and so on the basis of surface rheology alone we might expect the casein emulsion to be much less stable. It may therefore be premature to generalize that interfacial viscosity is the predominant factor defining the stability of these emulsions. Presumably, in the case of the casein system, the surface charge density and/or the steric effect of the adsorbed protein are sufficient to overcome the tendency for droplets to flocculate or coalesce. Is there any likelihood of being able to determine a priori which particular effects will actually predominate in an emulsion containing a specified protein?

Authors: There seems little doubt that the combined mechanisms of electrostatic and steric stabilization are of paramount importance: what primarily determines stability is the strength of these interparticle forces in relation to other forces due to Brownian motion, gravity, fluid flow, etc. The problem is, of course, that at present we have almost no useful quantitative information on the strength of these interparticle forces for protein-coated droplets. That being the case, one is tempted to look towards other secondary factors, especially those which are amenable to direct experimental investigation. Ease of deformation of the adsorbed protein layer (surface rheology) is one such secondary factor. It is not our intention, however, to imply that there is any direct causal relationship between the low-shear surface shear viscosity and the high-shear orthokinetic emulsion stability. What we are rather modestly suggesting is that, for this emulsifier + β-lactoglobulin emulsion system, there may be some link between the molecular interactions controlling orthokinetic destabilization and those controlling the surface shear viscosity.

Emulsion properties depend on adsorbed layer molecular properties in ways which we do not properly understand. This leads sometimes to ambiguities and apparent contradictions of interpretation. Binding of calcium ions to adsorbed casein leads to both increased flocculation (implying an increased coalescence rate) and increased surface viscosity (implying a decreased coalescence rate). From the results in Figure 8, we can conclude that in this instance the negative effect of flocculation predominates. Whether it can be regarded as a general rule remains to be seen.

D. G. Dalgleish: The emulsions described in this paper all contain a fixed amount of protein (0.4 wt%). We are finding that the surface composition and properties of similar emulsions depend very much on the protein concentration. For example, we can make stable caseinate emulsions [Fang H, Dalgleish D G (1993) J. Colloid Interface Sci., in press] with Γ ranging from 1 to 3 mg m⁻² with interfaces whose composition can then be changed. Are the authors certain that it is simply the ratio of emulsifier to protein that is important in defining the properties of the emulsions, or is the absolute amount of protein important as well? Our experience suggests the latter. So we would welcome any information that the authors could give on how variation of the protein concentration affects their results.

Authors: The reviewer's findings are certainly interesting. We ourselves have not made systematic measurements as a function of protein concentration. A fixed amount is chosen in order to standardize our formulation conditions thereby facilitating comparison between different proteins, surfactants, oils, etc. The actual amount used is enough to produce a fine stable emulsion under our single-pass homogenization conditions. To maximize the precision of the competitive adsorption experiments, we try to avoid making emulsions with a large excess of unadsorbed protein. Where essentially all of the available protein is adsorbed (i.e. in our pure β-casein emulsions with estimated monolayer coverages of 2–3 mg m⁻²), we believe that the surfactant/protein molar ratio R is a useful measure of the relative amount of emulsifier required for protein displacement, especially when comparing different emulsifiers added to emulsions made under similar conditions. Were we to be able to carry out experiments on identical β-casein emulsions except with a lower surface coverage (~1 mg m⁻²), then it would be my guess that the R value for complete protein displacement might turn out to be rather higher. This view is based on the observation that protein becomes fully displaced when the amount of available water-soluble surfactant becomes large enough to make a saturated surfactant monolayer at the oil–water interface (~1.35 mg m⁻² for C12E8). Another possible variable to use instead of R in the protein displacement isotherm could be “relative available surfactant surface coverage”, i.e. amount of surfactant present relative to that for a saturated monolayer. This variable might be more appropriate in systems where there is multilayer adsorption or where a large proportion of protein remains unadsorbed.

A. Gaonkar: An interesting aspect of this study is the effect of calcium ions on orthokinetic stability of emulsions made with caseinate. Producing flocculation seems also to promote coalescence. While it seems logical that flocs would be sites for coalescence due to the close proximity of the droplets, I would like to know if interfacial viscoelasticity could play any role in counteracting coalescence in a flocculated system. Is
there a way to estimate the amount of interfacial elasticity required to counteract coalescence within flocs? Do the authors have any surface rheological data relating to the interaction of calcium with caseinates that might indicate an alteration in surface rheology which could influence the coalescence rate in flocs?

**Authors:** Experimental data is available [Hunt J A (1992) Ph.D. thesis, University of Leeds] to show that the surface shear viscosity of \(\beta\)-casein adsorbed at the oil–water interface increases substantially in the presence of calcium ions \((\geq 10 \text{ mM})\). This behaviour is explicable in terms of denser packing and more extensive intermolecular interactions in the adsorbed casein layer due to local charge neutralization and ion-bridging as calcium ions bind strongly to oppositely charged phosphoserine side-chains. (Similar behaviour is also found with the highly phosphorylated egg-yolk protein phosvitin.) The problem is that, for the caseinate/calcium system, the same interactions which cause increased interfacial viscoelasticity also tend to induce flocculation. So it is rather difficult to disentangle the two effects in any interpretation of the orthokinetic stability results for this set of systems. What could perhaps give some insight would be comparison with experiments on emulsions flocculated in other ways (e.g. by pH change, enzymic hydrolysis, alcohol addition, etc.), where, for approximately the same degree of flocculation, the influence on the surface viscoelasticity of the adsorbed protein layer might be quite different. On the question of how to estimate the amount of interfacial elasticity required to resist coalescence within flocs, maybe some progress could be made by trying to correlate the high-shear coalescence rate with careful (low stress) measurements made at very low shear-rates. The latter are known to be rather sensitive to interdroplet interactions and floc strengths within weak gel-like flocculated structures.

**E. H. Lucassen-Reynders:** I have a difficulty with the proposed mechanism of protein partition into reversed micelles. If the concentration of lipophilic surfactant is high enough for such micelles to form, then it should be high enough also to displace some protein from the interface, but there is no sign of this in the initial protein surface concentrations given in Figures 2 and 3. Values of the critical micelle concentration (cmc) of \(\text{C}_{12}\text{E}_2\), the total surface area, and the equilibrium protein concentration should be specified for a judgement on this point to be possible.

**Authors:** The emulsion produced with pure \(\beta\)-lactoglobulin has a specific surface area of \(1.7 \text{ m}^2 \text{ g}^{-1}\). The protein concentration determined in the serum phase corresponds to 40% of the total protein present (i.e. 60% is adsorbed). In the emulsion with 0.1 wt% \(\text{C}_{12}\text{E}_2\), the proportion of \(\beta\)-lactoglobulin detected in the serum phase is lowered to 32% (i.e. 68% is associated with the oil droplets). Assuming that a saturated surfactant monolayer is reached at a \(\text{C}_{12}\text{E}_2\) surface concentration of 1.3 mg m\(^{-2}\), one can estimate that there is enough \(\text{C}_{12}\text{E}_2\) present in the emulsion made with 0.1 wt% \(\text{C}_{12}\text{E}_2\) to give about half monolayer coverage. At this level of lipophilic surfactant addition there is no significant change in inferred protein surface coverage in the “initial” emulsion compared with the surfactant-free system. However, the analysis procedure does take 15–30 minutes to carry out. Therefore one cannot rule out the possibility that roughly equal rates of protein displacement and partitioning into reversed micelles taking place simultaneously over this short timescale could be misinterpreted in terms of no net protein displacement into the bulk aqueous phase. The overall \(\text{C}_{12}\text{E}_2\) concentration is well above the cmc \((-10^{-3} \text{ wt\%})\), but the actual bulk phase \(\text{C}_{12}\text{E}_2\) concentration may be close to or even below the cmc if most of the surfactant present is adsorbed at the emulsion droplet surface. What the results point towards is some sort of attractive surfactant–protein interaction on the oil side of the interface. Whether it is really feasible (or necessary) to distinguish between surface complexation and partition into reversed micelles remains an open question.

**P. Walstra:** The authors suggest that droplets larger than 4 \(\mu\text{m}\) are disrupted. From what is known about the disruption of droplets in (isotropic) turbulence, it can be derived that this would need a power density of \(\sim 10^8 \text{ W m}^{-3}\). The emulsion heat capacity is \(\sim 4 \text{ MJ m}^{-3} \text{ K}^{-1}\). This then leads to heating of the emulsion by 25 K in 10 s, which is clearly impossible. Even if only a few per cent of the liquid were subject to the maximum power density, the heating rate would still be excessive.

**Authors:** The rate of energy dissipation in the emulsion during continuous shearing is estimated to lie somewhere between \(10^5\) and \(10^6 \text{ W m}^{-3}\). This agrees with an observed temperature rise of 10–20 °C. We interpret the relatively steady average droplet size reached at long times \((t > t_0)\) as indicating a dynamic balance between coalescence and droplet disruption processes occurring at equal rates. Indeed, this final steady-state average droplet size is roughly what one obtains if the bulk ingredients are mixed together coarsely at \(t = 0\) and then the coarse premix is emulsified by high-speed mixing over the same extended period of time. If the theory does not predict droplet disruption under these conditions, then presumably something is wrong with the theory.
A COMPARATIVE STUDY OF NORMAL AND HARD-TO-COOK BRAZILIAN COMMON BEAN (Phaseolus vulgaris): ULTRASTRUCTURAL AND HISTOCHEMICAL ASPECTS

Elisabeth Garcia¹, Franco M. Lajolo¹, Barry G. Swanson²

¹Dep. Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Caixa Postal 66355, CEP 05389, São Paulo, Brasil
²Present address: Dept. of Food Science and Technology, University of California, Davis, CA 95616-8598

Abstract

Legume seeds stored under high temperature and humidity develop a texture defect known as hard-to-cook (HTC). Structural and histochemical characteristics of normal and HTC beans (Phaseolus vulgaris) were studied after storing them at 5°C/40% relative humidity (RH) or 40°C/75% RH for 60 days. Cotyledonary cells of HTC beans showed contraction of the cell content, whereas the cytoplasm of normal seeds occupied the total cell volume. Cell walls of HTC beans appear more compact, showing smaller intercellular spaces. In normal beans the cell walls of adjacent cells had larger spaces between them. Pectic material of sections of hard beans stained more intensely than cell walls of normal beans. Preliminary results suggest that cell walls of hard beans contain more calcium than normal beans. HTC-and-old beans (6 years of storage) were observed under SEM in comparison to irradiated beans. Irradiation of beans caused softening of the seeds. The results confirm involvement of the cell wall-middle lamella in the hardening of bean seeds.

Key words: Legumes, hard-to-cook, ultrastructure, histochemistry, beans, Phaseolus vulgaris, textural defects.

Introduction

The softness of cooked beans is an important condition for consumer acceptance. Beans stored at high temperature and humidity develop a texture defect known as hard-to-cook (HTC), because they do not soften during regular cooking. The deterioration of cooking and organoleptic attributes leads to economic losses resulting either from increased requirement for energy to achieve softening, or consumer rejection. In addition, HTC can decrease protein biological value of beans (Sgarbieri and Whitaker, 1982).

Involvement of different components of the cotyledonary tissue has been proposed to explain the development of HTC. Soft texture is associated with the ability of cotyledon cells to separate easily in cooked beans (El-Tabey Shehata, 1992). Some authors inferred the participation of pectin and phytin (Jones and Boulter, 1983a, b; Moscoso et al., 1984), phenolic compounds (Hincks and Stanley, 1987; Srissuma et al., 1989), and changes in starch and protein solubility (Molina et al., 1975; Hohlb erg and Stanley, 1987; Hussain et al., 1989). Changes at the structural level have also been reported (Rockland and Jones, 1974; Varriano-Marston and Jackson, 1981; Shomer et al., 1990). In spite of the knowledge accumulated on HTC, an adequate understanding of the physiological mechanism(s) has not yet been achieved.

The objective of this study was to compare structural and histochemical characteristics of normal and HTC seeds of the Brazilian Carioca cultivar of common bean (Phaseolus vulgaris) to obtain a clearer comprehension of the HTC phenomenon in situ. Specifically, we wanted to clarify the role of the middle lamella-cell wall in the HTC process.

Material and Methods

Material

Common bean (Phaseolus vulgaris, cv Carioca) seeds were provided by the Instituto Agronomico de Campinas-SP. Carioca beans have a cream background with tan stripes. Control samples were kept at 5°C/40% RH for 60 days.
relative humidity (RH) and the HTC samples at 40°C/75% RH for 60 days (accelerated hardening; Vindiola et al., 1986). Also a sample of beans referred heretofore as HTC-and-old was kept under room conditions for 5 to 6 years. A sub-sample of the beans stored for 6 years was irradiated with $^{60}$Co at a 10 krad dose prior to storage.

Methods

Light microscopy. After removal of the seed coat, beans were fixed in ethanol:formaldehyde:acetic acid:water (30:10:10:10) for 7 days, with three substitutions of the fixative solution. The samples were then embedded in paraffin and sectioned (5 μm).
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Figure 2. Scanning electron micrographs of cross-sectional view of dry common bean seeds: normal seed (Fig. 2a); HTC seed (Fig. 2b). Figure 2b illustrates the separation between the cytoplasm of HTC cotyledon cells and cell wall (see arrows). Bar = 6.67 μm (a); and 10 μm (b).

Sections were stained with a 1% methylene blue solution for 5 minutes to stain the pectic substances of the cell wall/middle lamella area (Kertesz, 1951). The excess dye was removed by successive washings with distilled water.

A 0.05% toluidine blue solution in acetate buffer pH 4.4 was also used for staining. After removal of excess dye by washing with water, the sections were air-dried and mounted in oil. Polycarboxylic acids (including pectic acids) are stained red and lignin and polyphenolics are stained green (Feder and O'Brien, 1968). Sections were viewed with a Nikon Labophot microscope.

Staining with 0.1% Congo red for 5 minutes was used to reveal β-glucans of the cell wall (Fulcher et al., 1984). After washing the sections to remove the excess dye, they were air-dried and mounted in non-fluorescent immersion oil. Sections were viewed with a Nikon Fluophot microscope using IF 400-500 and 580 W filters.

Calcium was detected by treating sections with chlorotetracycline (CTC, Sigma C-4881), and viewing with a Nikon Fluophot microscope (IF 385-425 and 530 W filters) between 5 and 30 minutes after staining with CTC. A second slide prepared in the same way was subsequently treated with ionophore A23187 (Sigma C-7522) and the emitted fluorescence was observed as previously described (Powell, 1987).

Scanning electron microscopy. For the observation of the hilum, micropyle and seed coat surface, whole beans were mounted on aluminum stubs using colloidal graphite cement, and sputter coated with a 300 μm layer of gold. To fracture beans for cross-sectional examination, bean seeds were soaked in water overnight and then fixed in glutaraldehyde/formaldehyde (3%/3%) in 0.05 M, pH 6.8 phosphate buffer for 24 hours. The samples were then rinsed three times with phosphate buffer (0.05 M, pH 6.8), and finally with water. Subsequently, the samples were dehydrated using a graded ethanol series (10%; 20%; 30%; 40%; 50%; 60%; 70%; 80%; 2 X 95%; 3 X absolute ethanol), and finally critical point dried (Humphreys, 1974). The dried cotyledons were fractured under liquid nitrogen, using a razor blade hit with a hammer. The fractures were then sputter coated with gold (300 μm) after mounting on aluminum stubs (as described for the whole beans). The samples were examined with a Hitachi S-570 scanning electron microscope at an acceleration voltage of 20 kV. Pictures were taken with Polaroid 55 (P/N) film.

Results and Discussion

Despite the vast literature published on HTC, there is still controversy about the causes of this phenomenon. A comparison between HTC and normal bean seeds through microscopy techniques could reveal structural changes leading to an identification of the causes of the HTC phenomenon.

General structural characteristics

Preliminarily, an external characterization of the
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Carioca beans was performed to compare this variety, widely cultivated in Brazil, with other common bean varieties described in the literature. Figures la and lb show micrographs of the hilum and the micropyle of the Carioca beans. These structural features were similar to the varieties examined by Agbo et al., (1987). Observations of normal and HTC beans revealed no obvious differences in the external structural features. Even HTC-and-old beans (5 and 6 years old) did not reveal any external differences, except discoloration, which is a known characteristic of hard beans (Burr et al., 1968; Garruti and Bourne, 1985; Vindiola et al., 1986). The seed coat is shown in Figures 1c and 1d. A transverse fracture of a cotyledon (Figure 1c), showing the multiple layers (palisade, sub-epidermal and parenchyma) of the seed coat, is similar to the description of Hughes and Swanson (1985). Figure 1d shows the external appearance of the seed coat. The micrographs of the mature Carioca bean seed coat are similar to the black bean sample examined by Hughes and Swanson (1985) 19 days after flowering. The mature black seeds have a different seed coat pattern. No differences in seed coat were detected among normal (soft) and HTC Carioca beans.

Longitudinal sections of beans were viewed under light microscopy (results not shown). Cotyledonal cell size varied greatly. Vascular bundles were distributed through the entire cotyledon. Cells contained several starch granules, which were round- or oval-shaped and varied in size.

Viewed by SEM, the cytoplasm of normal seeds occupied the total cell volume (Figure 2a). In contrast, HTC bean cotyledon cells showed an accentuated contraction of the cellular contents (Figure 2b). This confirmed the observations of Varriano-Marston and Jackson (1981).

Staining with Congo red

Histological sections of beans were stained with Congo red and observed under fluorescence microscopy (Figures 3a and 3b). Hard beans showed a more compact packing of the cells, whereas normal beans showed a better definition of cell wall separation and larger intercellular spaces (Figure 3a).

Hardened samples also showed a more compact cytoplasm and cell wall (Figure 3b). Walls of adjoining cells were not well differentiated: in some areas only one stained line could be seen, instead of the two lines visible between two adjacent cells in normal seeds (Figure 3a).

Staining with toluidine blue

Longitudinal cotyledon sections were stained with toluidine blue which reacts with polycarboxylic acids giving a red color (Feder and O’Brien, 1968). Cell walls of HTC beans exhibited a stronger coloration of the cell walls (compare Figures 3c and 3d). Taking into account the composition of plant cell walls (Selvendran, 1983), our observations indicate that the pectins are reacting with the dye with different affinities in normal and HTC beans. It is possible that more carboxylic groups are free in the hard sample leading to the stronger coloration observed in Figure 3d, as in the normal seeds probably the methanol-esterified form predominates. Shomer et al. (1990) also detected an intense staining of the cell wall in HTC beans. The stronger affinity of the cell wall for the toluidine blue staining suggests that a higher number of free carboxylic groups are available for cross-linking between pectin molecules. Our observation is in agreement with the decrease in pectin esterification reported by Jones and Boulter (1983b) and decrease in pectin solubility (Jones and Boulter, 1983a, b; Hentges et al., 1991).

Fluorescence with chlorotetracycline (CTC)

The participation of pectin in the hardening process could result from a prior de-methoxylisation step following pectinesterase action (Jones and Boulter, 1983b; Stanley and Aguilara, 1985). An increased amount of calcium and/or magnesium at the cell wall/middle lamella level would be a further indication of formation of insoluble pectates. The presence of these divalent cations was investigated using a specific fluorescent probe, CTC. CTC binds to calcium and magnesium producing a fluorescent emission (Powell, 1987). If, after CTC reaction the samples are treated with the ionophore A23187, the Ca-CTC fluorescence is suppressed and the Mg-CTC emission can be detected. Using this technique, we observed that calcium was more abundant than magnesium at the cell wall/middle lamella (results not shown).

Histological sections of soft and hard beans treated with CTC are presented in Figures 3e and 3f. HTC bean sections showed a higher fluorescence over the entire cell wall contour, while soft bean sections revealed more intense fluorescence, predominantly at cell wall corners. Unfortunately, it was not possible to measure the fluorescence intensity to get a precise comparison of the samples. The Ca-CTC fluorescence was detected in the cell wall and not in the intercellular space (Figures 3e and 3f). This suggests that calcium was bound to the cell wall matrix pectin or was cross-linking outer chains of pectins of the middle lamella.

Fluorescence resulting from treatment with ionophore A23187 was comparable in both soft and hard beans (micrographs not shown). Residual fluorescence (Mg-CTC) was mainly restricted to the corners between cells.

The middle lamella appears to play an important role in the hardening process. Soaking of HTC beans...
leads to mineral losses possibly as a consequence of increased membrane permeability (Varriano-Marston and Jackson, 1981; Jones and Boulier, 1983; Hincks et al., 1987; Srisuma et al., 1989; Hentges et al., 1991). Consequently, calcium ions released through the action of phytase upon phytic acid located in the protein bodies could cross the cell walls in higher amounts and eventually be bound by the free carboxyl groups of the pectins in the middle lamella. Carpita (1987) suggested that the cross-linking capacity of calcium favors the hypothesis that cell wall rigidity is controlled by the middle lamella pectin gel strength. Previous observations (Rockland and Jones, 1974; Varriano-Marston and Jackson, 1981; Phak et al., 1987; Shomer et al., 1990) showed that the middle lamella of hard beans resists solubilization during cooking, thus preventing cell separation.

**SEM of irradiated seeds**

Because Mancini-Filho (1990) had reported a drastic decrease in cooking time of irradiated Carioca beans, an SEM comparison was then performed of HTC and-old bean seeds (cooking time 520 minutes) and irradiated seeds (cooking time “zero” minute). HTC and-old beans (Figure 4a) revealed the same characteristics already described in Figure 2b: compactness of the cells and contraction of the cellular content. The cells of the irradiated sample were not broken during cotyledon fracturing, and were well separated; intercellular cohesion material was not detected. These general observations were very similar to those reported for cooked beans where middle lamella was solubilized (Hincks and Stanley, 1986). Probably the high irradiation dose used was responsible for the hydrolysis of pectic substances resulting in a drastic softening of the seeds. This would be an additional indication of the important role of the middle lamella in defining texture of cooked beans.

**Conclusions**

The following observations support the hypothesis that middle lamella-cell wall is involved in the development of HTC in beans through the interaction of minerals with pectin: (1) stronger staining with toluidine blue (higher level of free carboxyl groups) in hard beans; (2) calcium seems to be more abundant in the cell wall of HTC beans; (3) disappearance of the middle lamella in irradiated beans. Nevertheless, this seems to be one of the several mechanisms involved. A more detailed study of the cell wall/middle lamella is still required for an in-depth comprehension of the texture problems observed in legume seeds.

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**References**


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Figure 4. Scanning electron micrographs of cross-sectional view of dry common bean seeds. Fig. 4a shows HTC and old seed; Fig. 4b shows irradiated bean seed. Fracture of HTC and old bean seed exposes compacted cellular content and cell walls. Cotyledon cells of irradiated bean seed appear intact and separated. Bar = 50 μm.


Kertesz ZI (1951) The Pectic Substances. Inter-science, New York, p. 204.


Discussion with Reviewers

W.J. Wolf: In your discussion of irradiated beans you refer to "a cooking time of zero minutes". If they were simply soaked in water would they be as soft as cooked unirradiated beans?
Authors: The reference to cooking time of "zero" minutes is related to the type of measurement adopted. We used a modified Mattson cooking device (Jackson and Varriano-Marston, 1981). Bean seeds were arranged in the holes of the cooker, while the tip of a plunger with calibrated weight was resting on each seed. When the cooking device was completely assembled, it was immersed in a container with boiling water. When the plungers penetrated the seeds, the beans were judged as cooked. Cooking time is determined when half of the seeds were pierced by the plungers. In the case of the irradiated beans, as soon as the cooker was placed in contact with the water, the seeds were perforated, giving a cooking time of "zero" minutes. However, we should point out that those seeds had a "gummy" consistency; they were soft, but when smashed they did not give a paste.

L.B. Rockland: It would be of interest to know if starch gelatinization was apparent in the irradiated beans.
Authors: We did not study the isolated starch of irradiated beans further.

J.S. Hughes: Is there any information to indicate that "hardness" or the "hard-to-cook" phenomenon in legumes is a problem commonly encountered by consumers in Brazil?
Authors: The climatic conditions (high temperature and humidity) in many areas of Brazil favor the development of HTC. It is a common practice to soak the beans overnight and cook them (whole seed) using a pressure cooker. Unfortunately, as in many other Latin-American countries, we do not have statistical data on the different causes of post-harvest losses related to legumes.

J.S. Hughes: Were any structural differences noted between the HTC beans (40°C/75%RH) and the HTC old beans (5-6 years old)?
Authors: We did not notice any obvious structural differences between these samples. The only striking difference is the dark color of the seed coat in HTC seeds, and even darker in HTC old beans, compared with soft seeds.

J.S. Hughes: Do you have any explanation for why you were unable to fracture the individual cotyledonary cells in the irradiated beans?
Authors: We believe that there was dissolution of the middle lamella, rendering the tissue very flexible. As a consequence, the tissue was deformed upon contact with the razor blade, and the cellular contents were not exposed.

E. Varriano-Marston: The supposedly more intense dye left on the sections shown in Fig. 3d than in 3c may be due to washing techniques/time after staining rather than any real difference.
Authors: The results presented were repeatedly observed; we confirmed that the washing technique and time adopted were appropriate.

Authors: The Liu et al. (1993) paper reported that decreased solubility and thermal stability of intracellular proteins during storage limited water availability in the cotyledon during cooking, and reaffirmed the role of the middle lamella in bean hardening. Our study focussed on microstructural and histochemical techniques on the middle lamella and cell walls of hard-to-cook beans, concluding that greater concentrations of free carboxyl groups and calcium in the cell walls may be responsible for hardening. Liu et al. (1993) observed no structural differences between dry control and dry aged cowpeas; we observed contraction of the cell contents of beans stored at 40°C and 75% RH. Both papers recognize the inherent involvement of multiple physiological and structural mechanisms in legume hardening.