Heat-Induced Destabilization of Oil-in-Water Emulsions Formed from Hydrolyzed Whey Protein

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The emulsifying ability, heat stability, and coalescence stability of oil-in-water emulsions prepared with whey protein of varied degrees of hydrolysis (DH), and at varied protein contents, was studied. Whey protein hydrolysates (WPH) with a DH of 4% and 10% had poorer emulsifying ability than non-hydrolyzed whey protein concentrate (WPC), but were more heat stable. Increasing DH between 10 and 27% improved emulsifying ability and further improved the heat stability of the emulsion droplets. Increasing DH from 27 to 35% led to a big decrease in both emulsifying ability and heat stability. The quiescent coalescence stability of WPH emulsions was relatively good up to a DH of 27%. Above DH 27% emulsions become highly unstable. It appears that two mechanisms of instability are at work here. At low DH heat-induced denaturation and aggregation occur. In the DH range of 4−20% heat stability increases as protein globular structure is disrupted. At a DH greater than 27% we see a change from a hydrolysis-induced increase in heat-stability to coalescence instability, with a resultant large increase in emulsion breakdown during heating.

Keywords: *Emulsion; heat stability; hydrolysates; coalescence*

INTRODUCTION

Milk proteins, as well as being excellent emulsifiers, are also important as nutritional ingredients. They are particularly useful in this dual role when they are used to stabilize fat droplets in infant formulas, paediatric formulas, and enteral formulas (*1, 2*). Often milk proteins found in these formulations will be in a hydrolyzed form. That is, they have undergone enzymatic hydrolysis to break up their native structure and release peptides and amino acids. Hydrolysis can be beneficial for two reasons. The whey protein β-lactoglobulin has been linked with cows' milk intolerance in some infants, and its hydrolysis can alleviate this (*3, 4*). Hydrolysis produces small peptide fragments and/or free amino acids, and these may be more easily digested and absorbed in the gut of pre-term infants, or in hospital patients who are fed enterally (*5*).

The use of hydrolyzed protein in fluid enteral and infant formulas, however, can cause problems with the stability of the dispersed fat droplets. Highly hydrolyzed whey proteins are often used as these have a low allergenicity compared to that of intact proteins (*3, 4*). The small peptide fragments found in these products are poor at stabilizing emulsions because they form adsorbed layers with low surface viscosity. Therefore, the emulsions can be relatively unstable to coalescence (*1, 2, 6−8*). Several researchers have shown that mild hydrolysis of whey protein improves emulsifying and foaming ability (*9, 10*). Similar results have been observed for casein (*11*), soy protein (*12, 13*) and gluten (*14*). Studies have also shown that the emulsifying properties of hydrolysates depend on the enzyme type used (*15−17*). Presumably, this is because different enzymes are specific to different peptide bonds, and so they form peptide mixtures with differing chain lengths and hydrophobicities.

Other researchers (*6*) have studied whey protein hydrolysate (WPH) with a degree of hydrolysis (DH) in the range 8−45%. They found that emulsifying ability was greatest between 10 and 20% DH, and decreased sharply above DH 27%. No comparison was made with intact whey protein. Agboola et al. (*7*) studied the emulsifying properties of highly hydrolyzed WPH (DH 27%). They found that emulsion stability increased with increasing WPH concentration, and that for WPH concentrations below 2% the emulsions were unstable to coalescence (*7*).

Several studies have suggested that there is an optimum peptide molecular weight for emulsifying properties (*18−21*). Jeon et al. (*21*) fractionated a hydrolysate of cod frame protein using ultrafiltration with membranes of different molecular-weight cutoffs. They were able to demonstrate that emulsifying ability and stability was highest for permeate from a 10 kDa cutoff membrane. Other studies have shown that peptides require a chain length of more than twenty amino acids to have good emulsifying properties (*19, 20*). Agboola et al. (*7*) measured the peptide molecular weight/concentration profile in the adsorbed layer of DH = 27% WPH emulsions. They found that improved emulsion stability was linked to increased concentrations of high-molecular-weight peptides in the adsorbed layer. Emulsifying properties decrease greatly if the proportion of small peptides increases. Several mechanisms can be hypothesized to explain this. Increased hydrolysis may yield peptides of low surface activity that do not contribute to emulsification. An alternative

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explanation is that an increased degree of hydrolysis disrupts protein structure, and this will reduce the thickness and interfacial rheology of the adsorbed layer. This could result in reduced steric stabilization and increased coalescence. A third possibility is that increased hydrolysis may change the rate of adsorption of peptides, which in turn influences the droplet size distribution on homogenization. Hydrolysis also affects the denaturation and gelation properties of proteins. Studies (*22*−*24*) have demonstrated that limited hydrolysis improves the gelling ability of whey proteins. More extensive hydrolysis leads to a loss of gelling ability in whey protein (*25*) and in soy-protein (*1, 26*). Mahmoud (*1*) suggests that this loss of gelling ability results from a loss of hydrophobicity and an increase in charge repulsion in hydrolyzed protein molecules (*27*). To support this, it has been shown that hydrophobicity is positively correlated with gelation ability (*28, 29*).

Microbial stability is an important consideration in the processing of infant and enteral formulations, especially if they are to be fed to individuals who are particularly at risk from microbial infection. Therefore, these formulations are likely to be given a high-heat treatment, such as in-can retorting. To allow optimization of product stability without loss of sterility, it is important that we understand how protein hydrolysis alters the fat emulsion properties.

In this study we have investigated the effect of a wide range of DH on the stability of heated WPH emulsions. This will provide a better understanding of how hydrolysis-induced changes in protein structure can alter functional properties, and ultimately how this impacts product stability.

MATERIALS AND METHODS

Whey protein concentrate (WPC) and whey protein hydrolysates (WPH) of various degrees of hydrolysis (DH) were obtained from the New Zealand Dairy Board, Wellington, NZ. Imidazole (ultrapure) was purchased from BDH Chemicals, Poole, Dorset, UK. Sodium azide (Analar grade) was purchased from Sigma Chemical Company, St. Louis, MO, and soya oil was obtained from Andrews Industries, Takapuna, NZ. The manufacturers data for peptide composition and weight average molecular weight are listed in Table 1. Increasing hydrolysis leads to an increased proportion of small peptides and a decrease in mean molecular weight. Details of the manufacturing procedures and the specific identities of the enzymes used for hydrolysis are commercially sensitive information. It is known, however, that the individual hydrolysate products are manufactured at more than one site, and that the enzyme preparation used is likely to differ from site to site. As discussed earlier (*15*−*17*). peptide composition and functional properties of protein hydrolysates will vary with the enzyme used. Therefore, we might expect some variation in emulsifying properties from this.

Protein solutions were prepared by dissolving the appropriate mass of protein or hydrolysate (1, 2, 4, or 6 wt % in the final emulsion) and 0.001 wt % sodium azide in imidazole buffer (pH 7.0, 0.05 M) and heating for 1 h in a water bath at 40 °C. Oil-in-water emulsions (20 wt % soya oil) were prepared using a Jet homogenizer (Labplant Ltd, Huddersfield, UK) at a nominal homogenization pressure of 200 bar.

Aliquots of the emulsion (0.5 mL) were pipetted into 1-mL screw-top Eppendorf tubes. The Eppendorf tubes were placed in a plastic holder and immersed in boiling water. Tubes were removed at 20-s intervals over a period of 3 min. The removed tubes were cooled immediately in iced water. Immediately after cooling, the particle size distributions for the heated emulsions was measured using a Malvern Mastersizer E (Malvern Instruments, Malvern, UK), assuming a presentation code of 2NAD. This presentation code assumed a refractive index of the dispersed phase of 1.456, and of the continuous phase of 1.33. It was assumed that the droplet absorbance was 0.00. A portion of emulsion was retained unheated, and the particle size distribution was measured over an appropriate time period to enable estimation of the rate of quiescent destabilization. For unstable emulsions, measurement periods as short as a few hours were sufficient. For more stable emulsions, measurements were taken over several days. The two common destabilization mechanisms in emulsions are flocculation and coalescence. It is possible to distinguish between flocculation and coalescence by dispersing the emulsion in a solution of low-molecular-weight surfactant, such as sodium dodecyl sulfate (SDS). This displaces protein from the droplet surface and disrupts any flocs. For particle-sizing of our heated emulsions it was not desirable to break up flocs, because in doing so we would also break up heat-induced aggregates. For the particle-sizing carried out on unheated stored emulsions we did not attempt to distinguish between flocculation and coalescence, and so we did not disrupt emulsions with SDS prior to sizing. We do, however, believe that the main instability mechanism in unheated emulsions, particularly at high DH, is coalescence. Kinetic plots for coalescence assume first-order kinetics, whereas flocculation is a second-order process (*30*). In constructing the kinetic plots for quiescent stability we found the data to be represented better by a first-order plot than a second-order plot. It should also be noted that Agboola et al. (*7*) report that flocculation is not observed in emulsions made with WPH of 27% DH for protein concentrations of 4% and below. At a higher protein concentration (5%) the mean $d_{3,2}$ is reported to decrease by only 0.02 μm on dispersal in SDS solution. For these reasons we assume coalescence kinetics (first order) when analyzing the unheated emulsion particle-size data.

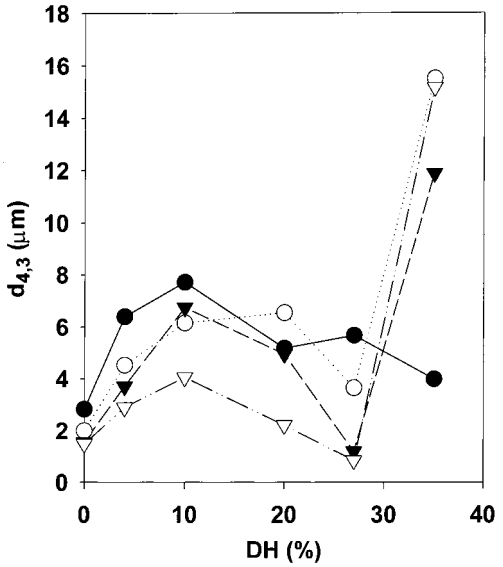

Figure 1. Weight-average diameter ($d_{4,3}$) as a function of degree of hydrolysis (DH) at four protein/peptide concentrations: ●, 1 wt %; ○, 2 wt %; ▼, 4 wt %; ▽, 6 wt %.

The kinetics of heat-induced aggregation of the emulsion droplets can be calculated using a method analogous to that used to study whey protein denaturation (*31, 32*). We have used this method previously to study whey protein emulsion droplet aggregation (*33, 34*).

The generalized rate equation for the change in emulsion droplet number (N) with time (t) is given by

$$-\frac{dN}{dT} = k_n N^n \qquad (1)$$

where k_n is the reaction rate constant and n is the reaction

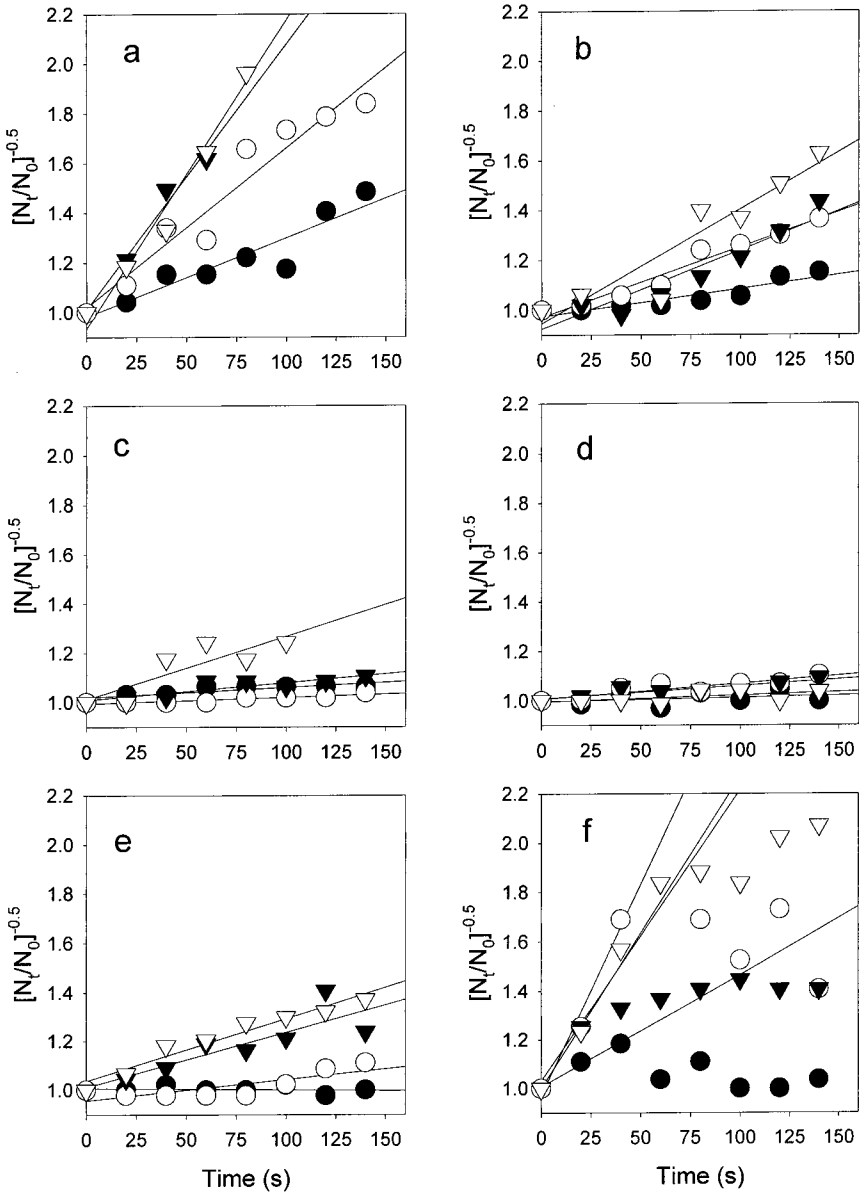

Figure 2. Kinetic plots assuming a reaction order of $n = 1.5$ for emulsions made with WPC and WPH of varied DH. (a) DH = 0% (WPC); (b) DH = 4%; (c) DH = 10%; (d) DH = 20%; (e) DH = 27%; (f) DH = 35%. The different symbols refer to protein contents of ●, 1 wt %; ○, 2 wt %; ▼, 4 wt %; ▽, 6 wt %.

Table 1. Percentage Peptide Composition of Whey Protein Hydrolysates (Manufacturers Data)

	percentage of total peptides in molecular weight range				
molecular weight range	DH 4%	DH 10%	DH 20%	DH 27%	DH 35%
<500	25	24	23	37.5	44
500−1000	4	11	13	23.5	15
1000−5000	19	24	27	37	27
5000−25000	45	41	37	2	14
> 25000	7	0	0	0	0
mean weight-average molecular weight	19900	1100	520	460	not available

order. A more useful equation is obtained by integrating eq 1 to give

$$\left[\frac{N_t}{N_0}\right]^{1-n} = 1 + (n-1)kt \qquad (2)$$

where N_0 is the initial droplet number, and N_t is the number after a time t. The parameter k in eq 2 is the apparent reaction rate, which is dependent on N_0, and is given by the equation

$$k = k_n N_0^{n-1} \qquad (3)$$

To be able to use eq 2 to determine the apparent reaction rate constant (k), assumptions have to be made about the reaction order n. For coalescence of emulsion droplets, $n = 1$ (first order) and eq 2 cannot be used. In this case an equation of the form

$$\frac{N_t}{N_0} = \exp(-kt) \qquad (4)$$

is appropriate (*30*, *31*). For a 1st order process a plot of $\ln(N_t/N_0)$ vs t should be linear with a slope of $-k$.

In previous studies we have found that heat-induced aggregation of whey protein stabilized emulsions is best described as a reaction of order 1.5 (*33*, *34*). This is consistent with the reaction order measured for β-lac denaturation; the major component of whey protein concentrates (*31*, *32*).

The relative particle number (N_t/N_0) can be estimated using the method suggested by Das and Chattoraj (*35*). This draws

on the relationship between emulsion droplet number, N, and the mean volume average droplet diameter, $d_{3,0}$ i.e.

$$\frac{4}{3}\pi\left(\frac{d_{3,0}}{2}\right)^3 N = constant \quad (5)$$

with $d_{3,0}$ obtained from the Mastersizer data. Equation 6 is valid only if the volume of emulsion droplets remains constant: i.e., if no oil separation occurs. The relative number of emulsion droplets can then be defined as

$$\frac{N_t}{N_0} = \left[\frac{(d_{3,0})_{t=0}}{(d_{3,0})_{t=t}}\right]^3 \quad (6)$$

In practice, kinetic plots of $(N_t/N_0)^{1-n}$ vs t may be nonlinear at longer heating times. This is due to the breakdown of the constant volume assumption. In this case k can be determined from the linear region of the plots.

Kinetic plots for $(N_t/N_0)^{1-n}$ vs t may also show a lag phase at short times before changes begin. This is due to a finite time for the emulsion to reach the heating temperature. Measurements of the come-up time using a thermocouple inserted into an Eppendorf tube were made. The time taken to reach the heating temperature (100 °C) was 35 s ± 1 s. When making kinetic plots, all particle size data acquired at heating times less than 40 s were discarded. When calculating N_t/N_0 the zero time was taken as the $t = 40$ s point.

RESULTS AND DISCUSSION

The emulsifying abilities of the intact WPC and WPH samples at different protein concentrations are presented in Figure 1. Here the $d_{4,3}$ (weight mean diameter), measured immediately after homogenization, is plotted vs DH. The $d_{4,3}$ is a measure of mean particle size that is more sensitive to the presence of large emulsion droplets that may be found, particularly, in an emulsion formed by poor emulsifiers. For each protein/hydrolysate sample four protein concentrations were used: 1%, 2%, 4%, and 6%. The emulsions tend to have a monomodal particle size distribution immediately after homogenization, becoming bimodal after heating or if coalescence occurs. The exception are emulsions made with WPH of 35%, which have a bimodal character after homogenization, and with the proportion of larger droplets increasing as protein content is reduced.

Increasing DH up to 10% decreases the emulsifying ability of the hydrolysates (increases $d_{4,3}$). For DH between 10% and 27% emulsifying ability increases, and then decreases sharply for DH 35%. For a peptide concentration of 1%, however, no increase in $d_{4,3}$ is seen on increasing DH from 27% to 35%. Agboola et al. (*7*) note that emulsions made with WPH of a high DH and a low peptide concentration can be highly unstable, with a portion of the oil phase not being homogenized. We also note the presence of free oil in emulsions made at DH 35% and low protein concentrations. Such a situation would lead to an apparent low $d_{4,3}$.

The results for emulsifying ability are in general agreement with those observed by Dalgleish and Singh (*6*), who report that a DH of 10−20% is required for maximum emulsifying ability, and that a DH of greater than 28% is detrimental to emulsifying ability. The decrease in emulsifying ability for hydrolysates with a DH of 4−10% may be due to the formation of peptide aggregates. It is known (*25, 36*−*39*) that lightly hydrolyzed whey proteins can form aggregates of hydrophobic peptides that affect solubility. The size of these aggregates would obviously depend on DH. It is possible that the presence of aggregated hydrophobic peptides reduces the emulsifying ability in the DH range 4−10%. Aggregated proteins are known to have lower emulsifying abilities than nonaggregated proteins (*40, 41*). This is because they tend to be less flexible and cannot spread rapidly to stabilize newly formed oil droplets. Protein aggregates can also lead to bridging flocculation, particularly if they are large (*40*). For hydrolysates of DH > 10% the peptides are reduced in size (Table 1), have a reduced tendency to aggregate, and any aggregates formed will be smaller. This is likely to improve their emulsifying ability. When DH exceeds 27% there is a large drop in emulsifying ability of the corresponding hydrolysate. Again, this is in agreement with the results of Dalgleish and Singh (*40*). Agboola et al. (*7*) have demonstrated that high-molecular-weight peptides are very important in determining the stability of WPH stabilized emulsions. This would suggest that for hydrolysates with DH over 27% the large reduction in emulsifying ability might be due to the absence of peptides of a molecular weight high enough to confer adequate coalescence stability.

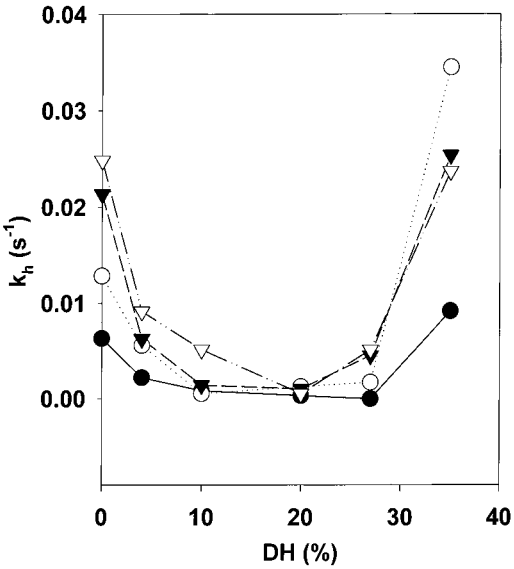

Figure 3. Plot of the rate constant for heat-induced aggregation (k_h) vs DH at four protein/peptide concentrations: ●, 1 wt %; ○, 2 wt %; ▼, 4 wt %; ▽, 6 wt %.

Because hydrolysis is known to affect whey protein gelation it is likely that the heat-stability of WPH stabilized emulsions will be similarly affected. Indeed Dalgleish and Singh (*6*) have demonstrated that emulsions formed from highly hydrolyzed whey proteins are unstable to heat-treatment by retorting at 120 °C. We should also note that the more hydrophobic peptides would be preferentially adsorbed at the droplet surface. Peptides remaining in the bulk phase are likely to be less hydrophobic, and this may also affect the heat stability of emulsions. In Figure 2 kinetic plots for the heat-induced aggregation of emulsion droplets are presented. In Figure 3 the apparent reaction rate constant (k_h, where the subscript h denotes heat-induced aggregation) is plotted as a function of the degree of hydrolysis (DH). The value of k_h is calculated from the slope of the kinetic plots in Figure 2. At DH below 20% k_h increases with increasing protein/peptide concentration. This is in agreement with our previous work (*33*) on whey protein stabilized emulsions. This observation is a simple consequence of kinetic theory, which implies

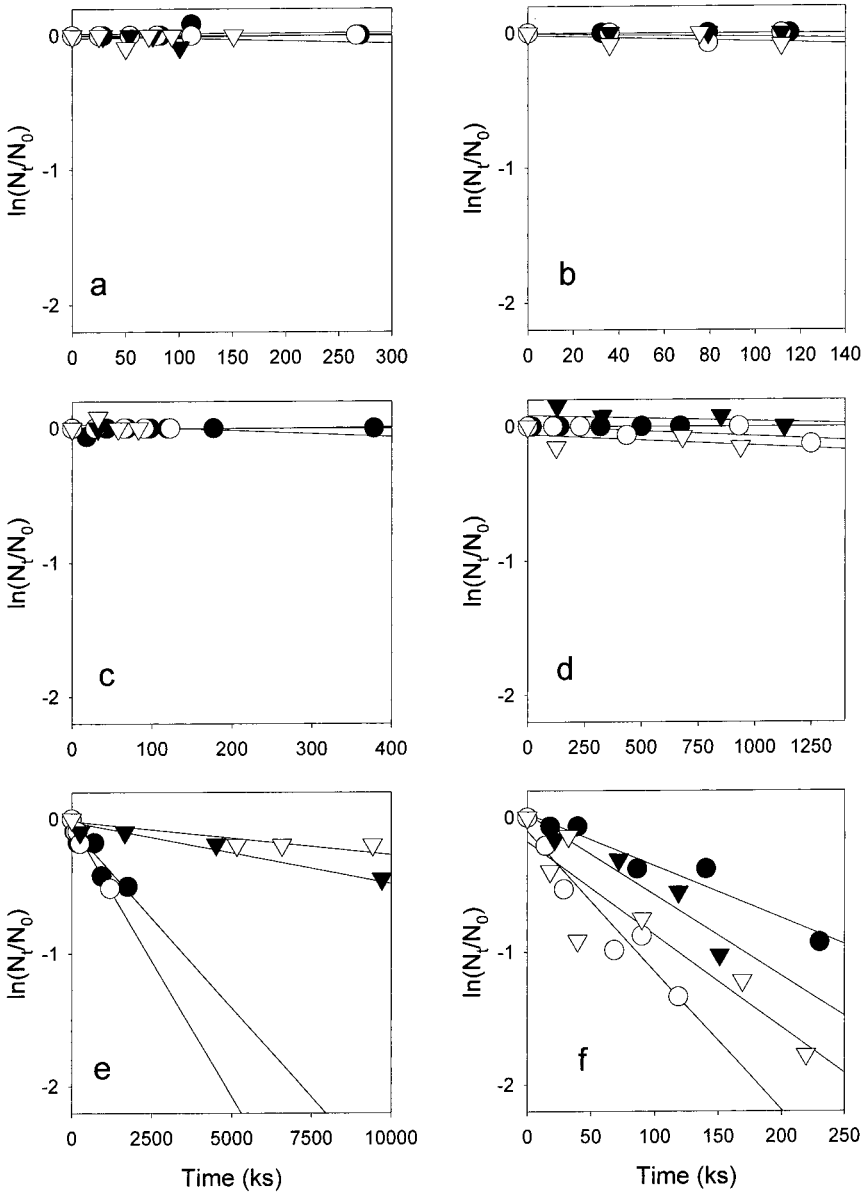

Figure 4. First-order kinetic plots for unheated, stored emulsions made with WPC and WPH of varied DH. (a) DH = 0% (WPC); (b) DH = 4%; (c) DH = 10%; (d) DH = 20%; DH = 27%; (f) DH = 35%. The different symbols refer to protein contents of ●, 1 wt %; ○, 2 wt %; ▼, 4 wt %; ▽, 6 wt %.

that for aggregation of whey protein the apparent reaction rate constant increases with increasing concentration of reactants (*31, 32*). As DH is increased heat stability increases, and the influence of protein concentration on k_h becomes less apparent. For DH = 20%, k_h appears to increase with increasing peptide concentration. It is likely that at peptide concentrations of 1% and 2% the emulsions become unstable, and determination of k_h becomes inaccurate because of rapid separation of large droplets. Indeed, it has been observed that under some homogenization conditions DH = 27% WPH exhibits free oil separation immediately after homogenization (*7*). This suggests some oil is not emulsified.

It is obvious that k_h depends strongly on DH. As DH increases up to 20% the rate of heat-induced aggregation decreases. If DH is increased to 27%, however, the rate of droplet aggregation increases above that of the emulsion made with WPH of 20% DH, but is still lower than that for emulsions made with native protein. For DH = 35% the emulsions are highly unstable, and the kinetic plot becomes nonlinear relatively quickly after heating is initiated. This is due to separation of the oil phase. The constant volume assumption necessary for eq 6 to be valid breaks down, and nonlinearity results in the kinetic plots. For DH = 35%, k_h is calculated from the initial slope of the kinetic plot. The dependence of k_h on DH is a result of the structural changes to the whey proteins (particularly β-lactoglobulin, β-lac) caused by hydrolysis. Whey protein comprises a mixture of globular proteins that have complex secondary and tertiary structures in solution (*42*). Heating a solution of whey protein to temperatures above about 70 °C is sufficient to denature them. Once denatured, there is a tendency for the protein molecules to self-associate into aggregates, and, if the concentration is high enough, to form gels. Hydrolysis of whey proteins leads to a loss of secondary and tertiary structure, as peptides are released (*43*). This is characterized by a reduction in the number of ionizable groups and exposure of hydrophobic groups (*44*). As well as altering emulsifying ability, this loss of structure also affects the ability of the protein to

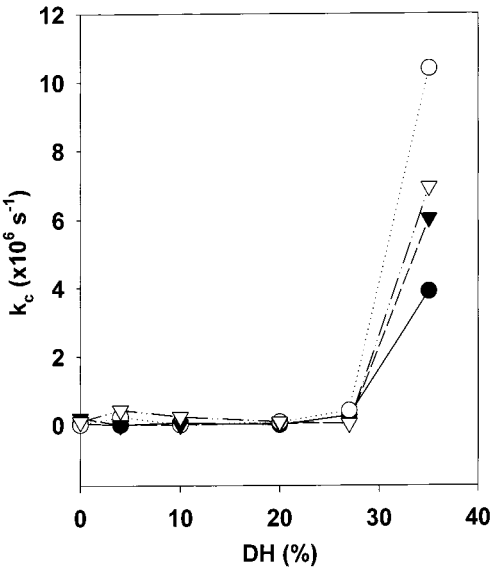

Figure 5. Plot of coalescence rate constant (k_c) vs DH at four protein/peptide concentrations: ●, 1 wt %; ○, 2 wt %; ▼, 4 wt %; ▽, 6 wt %.

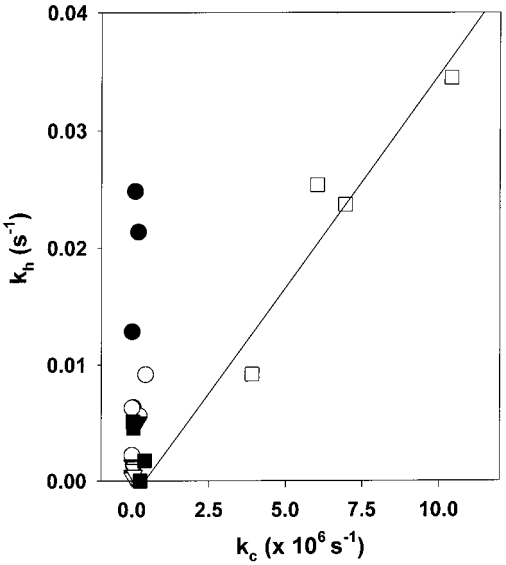

Figure 6. Correlation between k_c and k_h for emulsions made with WPC and WPH of varied DH: ●, 0%; ○, 4%; ▼, 10%; ▽, 20%; ■, 27%; □, 35%.

denature and gel. Limited hydrolysis of whey protein has been shown to improve emulsifying ability (*10*), and it also increases gel strength and gelling ability at neutral pH (*23, 24*). Presumably, limited hydrolysis alters the structure of the protein in such a way as to make it more labile to heat denaturation, possibly by lowering the conformational stability. More extensive hydrolysis of whey protein leads to a reduction in hydrophobicity and an increase in charge on the peptide fragments, and consequent loss of gelling ability (*1, 27*).

It is noticeable that the DH above which there is a large reduction in heat stability also corresponds to the DH at which hydrolysate emulsifying ability is substantially reduced. This suggests that the two phenomena are linked, and that the lower heat stability may be a result of a reduction in emulsion coalescence stability. To test this hypothesis we followed the change in mean particle size of stored unheated emulsions so as to deduce the rate of coalescence as a function of DH. Figure 4 is a first-order kinetic plot of the relative change in emulsion droplet number with storage time. The apparent coalescence rate (k_c) vs DH is plotted in Figure 5. It is obvious from Figures 4 and 5 that the emulsions have relatively high coalescence stability up to DH 27%. At DH = 27% and protein contents of 1% and 2% we begin to see a noticeable increase in k_c (Figure 5). This would appear to confirm our suspicion that the decrease in k_h at DH = 27% and low protein content may be due to a general reduction in emulsion stability. At DH 35% there is a large decrease in stability. Moreover, if we plot k_c versus k_h (Figure 6) there appears to be a close link between the two processes at 35% DH. The same trend is not apparent between the two rate constants for DH values of 27% and below. We should recognize, however, that the calculation of k_h at 35% DH is somewhat uncertain because of limited data, and the apparent correlation may be fortuitous.

Coalescence stability is linked to the ability of a protein or peptide to form a mechanically strong, viscous film at the droplet surface. Dickinson and Stainsby (*45*) demonstrated the link between coalescence stability and adsorbed protein interfacial viscosity, with protein having a higher surface viscosity forming more stable emulsions. Whey peptide fragments at the oil−water interface will almost certainly have a reduced surface viscosity compared to that of intact whey protein, and this can lead to reduced coalescence stability. As the degree of hydrolysis increases, progressively smaller peptide fragments are formed (Table 1). The globular structure of the proteins is disrupted and the released fragments behave more like flexible, random coil proteins such as the caseins. It is well-known that the interfacial viscosity of flexible proteins is significantly lower than that for globular protein (*46*). It is therefore a reasonable assumption that hydrolysis reduces a protein's interfacial viscosity. This in itself would lead to a reduction in coalescence stability. It should also be noted that the emulsions made with DH = 35% WPH have a larger particle size than emulsions made with WPH of a lower DH. Coalescence rate increases with increasing particle size (*30*) and so this would also contribute to the reduced stability.

One inconsistency, however, is that k_c for emulsions made with 35% DH WPH are some 2−3 orders of magnitude smaller than k_h for the same emulsions, and it is at first not apparent why heated emulsions made with 35% DH WPH are so unstable. To reconcile this, it should be remembered that k_c is measured at room temperature, whereas k_h is measured at 100 °C. During heating thermal motion will be greater, and this will lead to a higher collision frequency between emulsion droplets. If the emulsion droplets are prone to coalescence then this increased collision frequency will further increase k_h. This could explain the difference in magnitude of k_h and k_c at DH 35%.

In this work we have highlighted the fact that hydrolysis of whey protein leads to two opposing effects on the stability of heated WPH emulsions. At DH in the approximate range 4−20% hydrolysis has the effect of improving emulsion heat stability. Two factors could contribute to this. Breakdown of the whey protein native globular structure may reduce their ability to denature and aggregate thus increasing heat stability. Hydrolysis may also release hydrophobic peptides that have an increased likelihood of adsorption. The hydrophilic

regions of these peptides will not be available for aggregation, and this may also result in increased heat stability.

At DH above about 27% the peptide fragments produced are small (Table 1). These peptides are poor emulsifiers, and also impart poor coalescence stability on the emulsion droplets.

During heating, emulsions made with hydrolysates of DH > 27% are highly unstable to heat because of an increased tendency toward coalescence. The crossover between increased and decreased heat-stability through hydrolysis lies close to 27% DH in this work. Factors such as turbulent mixing combined with high heat, as may be found during rotary retorting, are likely to lead to a reduction in the DH at which coalescence instability would occur.

ABBREVIATIONS USED

$d_{4,3}$, weight mean particle diameter; DH, degree of hydrolysis; k_c, apparent coalescence rate constant; k_h, apparent reaction rate constant for heat-induced aggregation; WPH, whey protein hydrolysate; WPC, whey protein concentrate.

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