Enzymatic Hydrolysis of Milk Proteins Used for Emulsion Formation. 1. Kinetics of Protein Breakdown and Storage Stability of the Emulsions

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Hydrolysis of β-lactoglobulin and sodium caseinate in solution or while adsorbed at oil-water interfaces in emulsions formed from unmodified proteins was carried out with trypsin. The hydrolysis was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and dynamic light scattering. The stability during storage of the emulsions containing hydrolyzed proteins was also investigated by small-angle laser light scattering. The order of the hydrolysis of the individual caseins at the oil surface was $\beta > \alpha_s >>> \kappa$ compared with $\alpha_s > \beta > \kappa$ in solution. The overall rate of hydrolysis of sodium caseinate was the same whether adsorbed to the interface or in solution. Conversely, the rate of hydrolysis of β-lactoglobulin was much higher on the surface of emulsion than in solution. Emulsions formed from hydrolyzed protein solutions were less stable during storage than those formed from unmodified proteins and subsequently hydrolyzed or those formed with unmodified protein solutions.

Keywords: *Emulsions; casein; β-lactoglobulin; trypsin; peptides; emulsion stability*

INTRODUCTION

One of the major ways of modifying the functional properties of proteins is enzymatic hydrolysis. It is well established that proteins need to be amphiphilic for good emulsifying properties (Parker, 1988, Dalgleish, 1989). Not only do proteins have to possess both hydrophilic and hydrophobic moieties in their polypeptide chains, such moieties must be available for interaction with the aqueous and lipid phases, respectively. This property is controlled essentially by the protein flexibility. Therefore, as a result of their rigid structures and buried hydrophobic moieties, globular proteins are best modified before being used as emulsifiers (Hidalgo and Gamper, 1977; Shimizu *et al*., 1984; Griffin *et al*., 1984; German *et al*., 1985). Studies on enzymatic hydrolysis of more flexible proteins like caseins have also been carried out, sometimes as comparative studies to globular protein hydrolysis (Chobert *et al*., 1988; Van Hekken and Strange, 1993). Several methods have been employed to determine the emulsifying properties of the proteins after modification (Shimizu *et al.*, 1984; Chobert *et al*., 1988; Turgeon *et al*., 1992). However, the stability of such systems on storage has received little attention.

Over the last 10 years, several workers (Shimizu *et al*., 1986; Kaminogawa *et al.*, 1987; Dalgleish and Leaver, 1990; Persaud, 1995) have studied hydrolysis by a method in which protein was first adsorbed onto the oil−water interface during the formation of an emulsion and was then hydrolyzed by trypsin. These studies showed that hydrolysis of milk proteins was different in solution and in the adsorbed state. The same method has been used to describe aspects of the conformation of individual protein molecules, α_{s1}- and β-caseins, and β-lactoglobulin at liquid interfaces. However, no properties of these emulsions after hydrolysis were studied, and no comparisons have been made between such hydrolyzed emulsions and emulsions made with proteins that have been hydrolyzed.

We studied the rate and mechanism of the tryptic breakdown of β-lactoglobulin (β-lg) and of sodium caseinate, a mixture of the four naturally occurring caseins. Essentially, the two differ in their molecular rigidity. Tryptic hydrolysis was carried out on the proteins, which were either in solution or adsorbed to the surface of oil droplets. We also describe the formation of emulsions with protein solutions hydrolyzed to different extents and how these emulsions compare with the enzyme treated emulsions. Specifically, comparison of the size distributions and stabilities during storage were investigated by small-angle laser light scattering.

MATERIALS AND METHODS

Materials. Sodium caseinate was prepared in the laboratory from skim milk as previously described (Agboola and Dalgleish, 1995). The 2-mercaptoethanol was obtained from Fisher Chemicals (Mississauga, ON, Canada), and β-lg, TPCK-trypsin (type XIII), and the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Protein Solutions. Solutions containing 2% β-lg or caseinate were prepared by dissolving the freeze dried protein in 20 mM imidazole/HCl buffer (pH 7). The solutions were then filtered through a 0.22-μm Millipore filter (Millipore Canada Ltd., Mississauga, ON). Samples of κ-casein were separated from whole casein by the method described by Zittle and Custer (1963) and then depolymerized by adding 5 μL of 2-mercaptoethanol to 30 mg of κ-casein in a 3% (w/v) solution. This solution was dialyzed overnight against 20 mM imidazole/HCl buffer (pH 7), diluted to 2%, and filtered through a 0.22 μm Millipore filter.

Appropriate amounts of trypsin were carefully weighed and dissolved in 20 mM imidazole/HCl buffer (pH 7) and added to protein solutions to give enzyme:substrate ratios of 1:25 and 1:1000 for solutions of β-lg and caseinate, respectively. Hydrolysis was allowed to proceed at ambient temperature (25 ± 2 °C) for up to 24 h for β-lg and 60 min for caseinate. Hydrolysis was terminated by adding 50 μg soybean trypsin inhibitor (as 1 mg/mL solution in imidazole/HCl buffer, pH 7) per milliliter of protein solution. Emulsions (20% soybean oil in water) containing 0.5 or 1% (w/v) protein were made from

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β-lg solutions hydrolyzed for 4 and for 24 h, and from caseinate hydrolyzed for 5 and 30 min. These emulsions were made with a Microfluidizer M110S (Microfluidics Corp., Newton, MA) as described earlier (Agboola and Dalgleish, 1996a). From previous observations (Hunt and Dalgleish, 1994), we know that under these conditions nearly all of the protein is adsorbed to the oil droplets in emulsions containing 0.5% protein; in the emulsions containing 1% protein, about three-fourths of the protein is adsorbed.

Hydrolysis of Adsorbed Proteins. Trypsin solution (1 mg mL^{-1}) was prepared in the imidazole buffer. To a 1-mL aliquot of emulsion made with unhydrolyzed caseinate or κ-casein, sufficient trypsin solution was added to provide an enzyme:substrate ratio of 1:2000. Hydrolysis of surface β-lg was essentially the same as in the caseinate system except that the ratio of the enzyme to the protein was 1:1000. The hydrolysis was allowed to proceed at ambient temperatures for periods ranging from 15 s to 1 h for caseinate-stabilized emulsions and up to 2 h for β-lg-stabilized emulsions. The reactions were stopped either by adding sodium dodecyl sulfate (SDS) and mercaptoethanol [during sample preparation for polyacrylamide gel electrophoresis (PAGE)] or by dilution when the particles were being sized to determine the extent of hydrolysis.

Analysis of Protein Hydrolysis. Aliquots of samples (300 μL emulsions or 150-μL protein solutions) at varying levels of hydrolysis were treated with 250 μL of 20% SDS, 100 μL of bromophenol blue, and 100 μL of 2-mercaptoethanol in glass vials. The vials were transferred to a boiling water bath and the contents were stirred vigorously for 5 min. After cooling to room temperature, $\approx$3 μL from each vial were loaded onto a polyacrylamide gel (20%) in a PhastSystem (Pharmacia LKB Ltd., Baie d'Urfé, PQ) according to the manufacturer's instructions. The protein bands were stained with Coomassie blue indicator, and the gels were allowed to dry for 24 h. The gels were then quantified with a laser densitometer (Hunt and Dalgleish, 1994).

Measurement of Particle Diameters. About 2 μL of each emulsion sample (unmodified, hydrolyzed to various degrees, or formed with hydrolyzed proteins) was pipetted into a cuvette containing $\approx$3 mL of filtered (0.22 μm) imidazole/HCl buffer at pH 7. The particle size was then determined by dynamic light scattering (DLS) with a Malvern Instruments 4700 optical system attached to a 7032 correlator (Malvern Instruments, Inc., Southboro, MA). Measurements were made at a scattering angle of 90°, and the diffusion coefficients and hydrodynamic diameters of the particles were calculated by the method of cumulants (Koppel, 1972), assuming that the particles were spherical and obeyed Stokes law. Repeated (triplicates) sets of 10 individual DLS runs were made for each sample, using appropriate sample times that gave good correlation functions (usually $\approx$30 μs).

To determine the stability of different emulsions to storage, the particle size distribution and average particle sizes of the emulsions were determined each day for 7 days by light scattering with a Malvern Instruments Mastersizer X. The presentation factor was 0303 (i.e., refractive index and absorption of the emulsion particles of 1.414 and 0.001, respectively). The emulsions were stored in the refrigerator at $\approx$4 °C. All experiments were carried out in triplicate and the average value is reported. The standard deviation ranged between $\pm$ 0.01 μm for unmodified emulsions and $\pm$0.35 μm for emulsions made with solutions of 0.5% β-lg, at the extreme of aggregation.

Measurement of ζ-Potentials of the Emulsion Droplets. The surface charges (ζ-potentials) of the particles in the emulsions were measured with a Malvern Instruments Zetasizer 4 attached to a Malvern series 7032 correlator. Measurements were made in the stationary layer (14.6% from the cell wall), where the electroosmotic effect is minimized. Emulsions, diluted 1: 2000 in deaerated imidazole buffer, were introduced into the measurement cell and the electrophoretic mobility of the particles was measured. Sets of 10 measurements were made on each sample and the results were averaged. Holding the diluted emulsions for up to 30 min under quiescent conditions before measurement did not result in any significant ($p > 0.05$) change in the value of electrophoretic mobility. The ζ-potentials were calculated from the electrophoretic mobilities by with the Henry approximation of Hückel's equation, setting the value of $f(\kappa a)$ to 1.5 (Hunter, 1981).

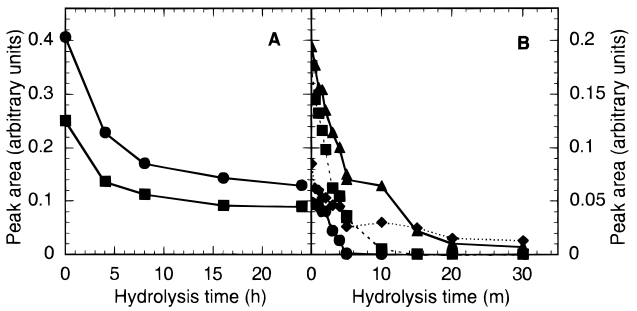

Figure 1. Breakdown of intact proteins in solution during hydrolysis with trypsin. (A) in (■) 1% or (●) 2% β-lg solution; (B) in 2% caseinate solution, showing the breakdown of the individual caseins [(●) α_{s1}-casein; (■) α_{s2}-casein; (▲) β-casein; (◆) κ-casein]. Note the different time scales for the breakdown of the β-lg and the caseins.

RESULTS AND DISCUSSION

Protein Hydrolysis. The rates of hydrolysis of the proteins, estimated by SDS-PAGE, are shown in Figure 1. Despite the high ratio of trypsin to β-lg, the substrate protein was very resistant to hydrolysis, especially in the latter half of the reaction (Figure 1A). The protein was >50% depleted after 8 h, and was reduced to $\approx$33% of the original level after 24 h. The resistance of β-lg to trypsin attack has been reported earlier (Antilla *et al.*, 1990; Turgeon *et al.*, 1992) and is presumably due to the presence of the covalent S—S bonds and considerable amounts of regular secondary structures (β-pleated sheets and α-helix). Because some of the arginine and lysine residues would be buried in this rigid structure, it is very likely that a number of the trypsin-susceptible sites would be rendered inaccessible.

The individual caseins in the caseinate solution were hydrolyzed at different rates (Figure 1B). Both α_{s1}- and α_{s2}-caseins were extensively depleted within the first 5 min and were fully degraded during 10 min of hydrolysis. Comparatively, β-casein, and in particular κ- casein, were degraded more slowly in solution, with full hydrolysis of β-casein taking >20 min, and that of κ-casein taking >30 min. From the primary structures of the individual caseins (Swaisgood, 1992), the content of the basic amino acids lysine and arginine are 30, 20, 15, and 14 for α_{s2}-, α_{s1}-, β-, and κ-casein, respectively. This variation in content of amino acids may partly explain the different rates of reaction, although β- and κ-caseins would be expected to behave similarly. The resistance of κ-casein could also arise from its ability to polymerize via S—S bonds (Swaisgood, 1992), which can increase the resistance of proteins to enzymatic attack (Antilla *et al.*, 1990; Turgeon *et al.*, 1992); β-casein also polymerizes in solution, which also changes its susceptibility to trypsinolysis (Leonil *et al.*, 1988). The caseins were hydrolyzed much more rapidly than was β-lg in these experiments, especially in view of the low enzyme: substrate (E/S) ratio of 1:1000 for the caseinate solution. This result is presumably because in caseinate, most of the bonds between lysine and arginine residues are accessible to trypsin, in contrast to the more structured β-lg.

Trypsinolysis of emulsified β-lg was much faster than in solution, being virtually complete in 2 h even at a

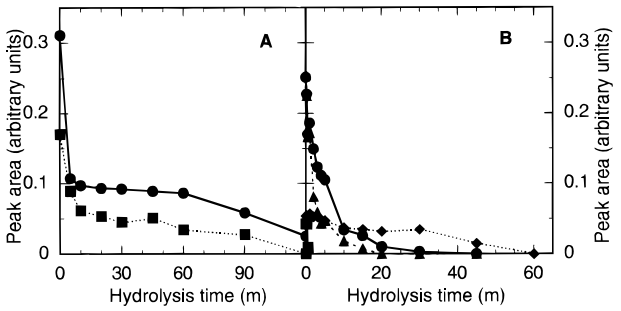

Figure 2. Breakdown by trypsin of intact proteins adsorbed to the oil−water interface in emulsions (20% w/v soya oil). (A) emulsions formed with (■) 0.5% β-lg or (●) 1% β-lg; (B) emulsions formed with 1% caseinate (symbols are same as in Figure 1). Note the different time scales for the breakdown of β-lg and caseins in this figure and in Figure 1.

much lower E/S ratio of 1:1000 (Figure 2A). The rate of hydrolysis was highest within the first 10 min, during which >50% of the protein was hydrolyzed; after this the rate fell considerably. Adsorbed β-lg may be more easily digested than in solution because of changes in conformation during adsorption, so that some hydrophilic residues that were hitherto buried would be expected to be exposed to the aqueous phase at the oil−water interface. Denaturation is known to accompany adsorption of globular proteins on the interface (Shimizu *et al.*, 1981; Corredig and Dalgleish, 1995). Thus, the flexibility of β-lg and therefore accessibility of trypsin-susceptible bonds will be somewhat improved on the surface because of the combined effect of conformational changes and denaturation.

As in solution, the adsorbed casein fractions were hydrolyzed at different rates (Figure 2B). In this case, however, β-casein was the most susceptible, followed by the α_s- caseins, and their trypsinolysis was most rapid within the first 5 min. On the other hand, κ-casein was stable to hydrolysis for >30 min, (i.e., until the hydrolysis of the α_s- and β-caseins was completed). This result prompted us to study the hydrolysis in emulsions made with purified κ-casein rather than the caseinate mixture. This κ-casein was depolymerized to facilitate emulsification because of the reported difficulty in making emulsions with polymerized κ-casein (Dickinson *et al.*, 1987). In these emulsions, hydrolysis of the protein started as soon as trypsin was introduced and was also most rapid during the first few minutes. The rate of hydrolysis was, however, still low compared with what had been measured for the other adsorbed caseins.

The results for hydrolysis of adsorbed caseins suggest that the rate of hydrolysis was strongly dependent on the accessibility of the casein fraction to the trypsin in solution. According to Dalgleish (1990), β-casein protrudes furthest from the interface into the aqueous phase (as much as 15 nm) when adsorbed on polystyrene latices, followed in size by the α_{s1}-casein (10 nm). Walstra *et al.* (1981) have shown that the apparent protrusion of κ-casein from the surface of casein micelles is of the order of ≈5 nm. The fact that, in the emulsions, the hydrolysis of κ-casein on the interface did not start until all the other casein fractions have been depleted suggest that the κ-casein may be buried among the other caseins on the interface. However, the relative resistance of κ-casein (as part of caseinate) to digestion by trypsin at the interface could also have been due to its polymerization via disulfide bonds. Also, κ-casein has the least number of trypsin-susceptible bonds of all casein fractions, the majority of which are within the hydrophobic *para*-κ-casein moiety, which is likely to be involved in the adsorption process. This last effect may contribute to the low rate of hydrolysis even when only κ-casein was present on the interface.

The rate of hydrolysis of the proteins on the surface of the emulsion was highest within the first few minutes, after which the rate gradually fell. Changes in conformation may be responsible for the lag in the rate of hydrolysis after an initial period of rapid digestion. Leaver and Dalgleish (1990) reported a similar observation on the hydrolysis of interfacial β-casein. It is thus suggested that the high initial rate of hydrolysis was due to the hydrolysis of the more accessible bonds. Subsequently, the remaining trypsin susceptible bonds were hydrolyzed more slowly because there had to be further changes in conformation before these remaining bonds could be made available to trypsin. In addition, the method measured only intact protein; subsequent attack of the trypsin on peptide fragments was not measured. Also, the enzyme may autodigest after the first few minutes of hydrolysis, to reduce the amount available for the reactions.

Changes in Particle Size during Trypsinolysis. The emulsions formed with either native or hydrolyzed protein solutions were analyzed for changes in the average droplet size by DLS. As hydrolysis time increased, the sizes of the particles (hydrodynamic diameters) decreased until they reached level values after 40−45 min of hydrolysis. The changes in diameter were 15 nm for the emulsion formed with 1% caseinate and ≈9 nm for the one formed with 0.5% caseinate. This difference has been explained by the need, when protein is limiting, for the protein molecules to stretch to cover the surface of the oil droplets, whereas at the higher concentration (1%) of caseinate, sufficient protein molecules are present to cover the oil droplet surface without stretching (Fang and Dalgleish, 1993). The lower concentration of protein thus gives a thinner layer. The change in size during trypsin treatment in the emulsion formed with 1% caseinate was somewhat smaller than the 20 nm change reported by Fang and Dalgleish (1993) possibly because they added trypsin to previously diluted emulsion whereas in this study, the enzyme was added to a concentrated emulsion. Extension of the incubation time beyond 60 min in this study did not result in further size reduction.

Compared with caseinate, emulsions stabilized by β-lg did not show any significant change in the sizes of the emulsion droplets during hydrolysis. This agrees with an earlier study (Dalgleish and Leaver, 1991) on the adsorption of β-lg to polystyrene latices. It was therefore assumed that the protein did not protrude significantly from the oil surface into the aqueous phase.

Hydrolysis of κ-casein-stabilized emulsions gave different results from the other protein stabilized emulsions. As shown in Figure 3, there was an initial reduction (≈4 nm for the emulsion formed with 0.5% protein and 9 nm for that formed with 1% protein), after which the emulsions aggregated. The emulsion formed with 0.5% κ-casein was less stable than 1% κ-casein-stabilized emulsion because visible particles began to appear after ≈20 and 40 min, respectively. The difference between these and the other emulsions (stabilized by caseinate and β-lg) was presumably related to the types of peptides remaining on the interface after tryptic attack. In caseinate and β-lg systems, the peptides remaining on the interface must be predominantly hydrophilic, whereas they were hydrophobic in the case of κ-casein. The 9-nm reduction in the size of the 1%

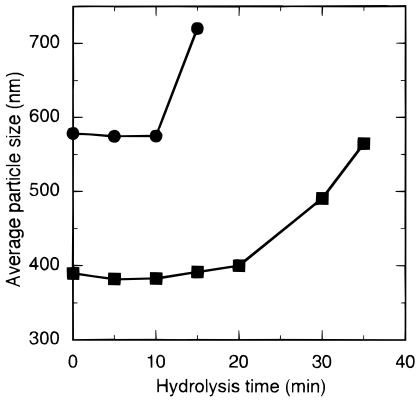

Figure 3. Aggregation of κ-casein-stabilized emulsions (20% w/v soya oil) during hydrolysis of the adsorbed protein with trypsin for emulsions containing (●) 0.5 and (■) 1% protein.

κ-casein emulsion is very close to the 10 nm reported by Walstra *et al.* (1981) for casein micelles during attack by chymosin. This similarity may indicate that tryptic attack on adsorbed κ-casein started close to the $Phe_{105}-Met_{106}$ bond which is cleaved by chymosin. There are three lysine residues in this region, at positions 111, 112, and 116. Attack at any of these sites means that the mainly hydrophilic C-terminal of the κ-casein will be removed from the interface, leaving the hydrophobic moiety, similar to *para*-κ-casein. This reaction will lead to aggregation of the particles, analogously to the renneting reaction in milk. The emulsion containing 0.5% κ-casein was less stable to trypsin attack because the depletion of the hydrophilic coat would be completed earlier. Also, it is possible that the emulsion stabilized by 0.5% κ-casein was less stable than that containing 1% κ-casein because of reduced protein coverage and different conformation of the adsorbed protein.

An attempt was also made to further hydrolyze emulsions made with incompletely hydrolyzed protein solutions, to determine whether there existed measurable protrusion into the serum phase, especially in the emulsions made with hydrolyzed caseinate solution. The changes in the hydrodynamic sizes of the emulsion droplets were very low and were within experimental error (±2.5 nm). In caseinate solution, the amounts of intact casein molecules were very low even after only 5 min of hydrolysis. Our results on the particle diameters show that any intact casein molecules did not protrude greatly from the interface. Most of the products of caseinate hydrolysis were likely to be short chain length peptides that did not protrude far into the serum phase.

Formation of Emulsions. The size distribution of emulsions formed with hydrolyzed protein solutions are shown in Figure 4. The emulsions formed from hydrolyzed β-lg solution were very different from those formed from native β-lg; they contained greater concentrations of particles with diameters of $>1\ \mu$m. Longer hydrolysis times of the β-lg before emulsion formation gave emulsions containing significantly ($p < 0.05$) larger particles and those formed with 0.5% protein had bimodal size distributions. The average particle sizes in the emulsions also depended on the concentrations of surfactants, as has been established previously (Tornberg, 1978; Parker, 1988). The results clearly demonstrate that the hydrolysis of β-lg by trypsin caused a decrease in the emulsifying power of the protein, presumably because small peptides formed have lost the capacity to interact with both aqueous and nonaqueous phases (Turgeon *et al.*, 1992). A peptide with very long chain length has a greater probability of having both hydrophilic and hydrophobic moieties on the same unit. It is also possible that the greater emulsifying power of the higher concentration of hydrolyzed β-lg arose from the presence of quantities of unhydrolyzed protein.

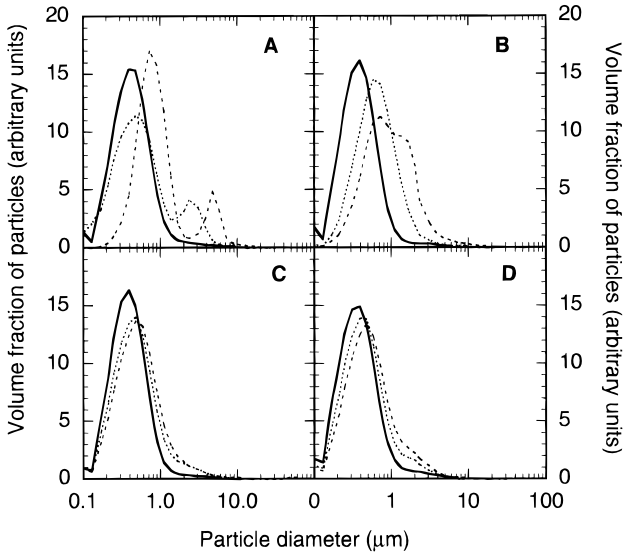

Figure 4. Distribution of particle sizes in emulsions measured the same day that they were made. The emulsions were prepared from unhydrolyzed (full lines) or hydrolyzed protein solutions. Panels A and B are for emulsions containing 0.5% and 1% β-lg, respectively, after trypsinolysis for 4 h (dotted line) or 24 h (broken line). Panels C and D are for emulsions containing 0.5% and 1% caseinate, respectively, after trypsinolysis for 5 min (dotted line) or 30 min (broken line).

These results, showing that the emulsifying power of β-lg was reduced by hydrolysis with trypsin, are different from what was seen by Shimizu *et al.* (1984), where reduction of the disulfide bonds in β-lg by mercaptoethanol resulted in increased emulsifying power. The difference arises because trypsin acts not simply to loosen the rigid structure of β-lg, but breaks up the regions that are important for emulsification. Disulfide bonds are of course unaffected by trypsinolysis, and we did not test the effect of adding mercaptoethanol after hydrolysis. Kato *et al.* (1983) have also reported reduced emulsifying power for denatured β-lg. It seems that the seemingly conflicting observations about the effect of hydrolytic modification on the emulsifying ability of β-lg reviewed by Dalgleish (1989) may be related to the specificity of the hydrolytic agent and the extent of the hydrolysis.

Emulsions formed with hydrolyzed caseinate maintained the monomodal size distribution (Figures 4C,D), but their particle sizes were significantly larger ($p < 0.05$) than those in emulsions made with unmodified caseinate and increased with the time of hydrolysis. So, again, the native protein was superior to the hydrolyzed form in emulsifying capacity. Compared with hydrolyzed β-lg, however, the hydrolyzed caseinate was a better emulsifier (i.e., the caseinopeptides were more surface active than the peptides obtained from β-lg). It is possible that there was synergism between the caseinopeptides as suggested by Lee *et al.* (1987) for peptides from α_s-caseins.

Stability of the Emulsions during Storage. The effect of storage time (1−7 days) on the size distributions of typical emulsions formed with hydrolyzed protein solutions is shown in Figure 5, and the average particle sizes of all types of emulsions are shown in Figure 6. Emulsions formed with hydrolyzed caseinate were all

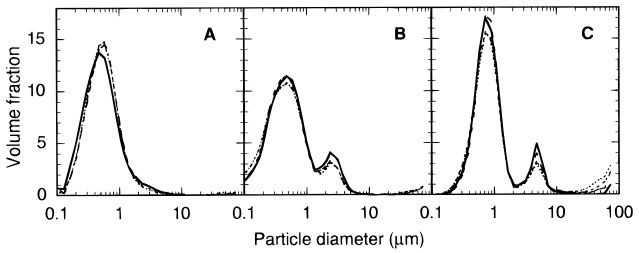

Figure 5. Effect of storage time (up to 7 days) on the particle size distribution of emulsions formed with hydrolyzed protein solutions. Full lines represent fresh (day 1) emulsions. The emulsions contained (A) 0.5% caseinate hydrolyzed for 30 min; (B) 0.5% β-lg hydrolyzed for 4 h; and (C) 0.5% β-lg hydrolyzed for 24 h. Changes were generally small, but there was significant appearance of large aggregates in panel C. See text for details.

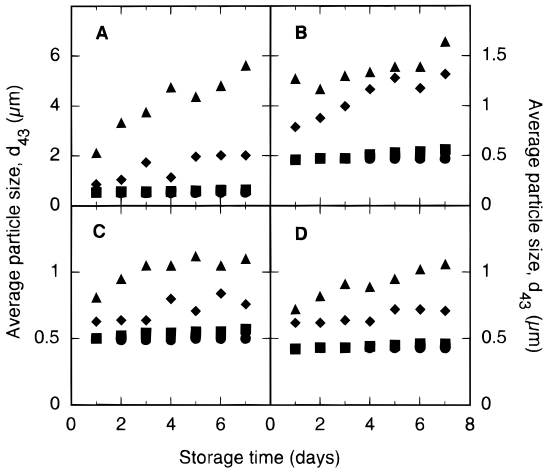

Figure 6. Comparison of the average particle sizes of emulsions formed with native proteins (■), emulsions formed with native proteins and then hydrolyzed (●), and emulsions prepared from hydrolyzed protein solutions (◆ and ▲). Panels A and B show emulsions based on 0.5 and 1% β-lg, respectively, with hydrolysis for (◆) 4 and (▲) 24 h. Panels C and D show emulsions based on 0.5 and 1% caseinate, respectively, with hydrolysis for (◆) 5 and (▲) 30 min.

fairly stable, although there appeared to be a very small population of very large particles (between 40 and 80 μm) after ≈2 days of storage in the emulsion formed with 0.5% caseinate that had been hydrolyzed for 30 min (Figure 5A). This aggregation must be related to the concentration of available amphiphiles on the interface because the least amount would be expected to be found in a system where the hydrolysis was the longest and the concentration the lowest.

The emulsions formed with hydrolyzed β-lg solutions were not stable with time, as shown in Figures 5B,C and 6A,B. The size distributions showed that the increase in the average sizes of the emulsions was primarily due to the increase in the number of particles whose sizes were >10 μm, which is in agreement with the observation by Dickinson and Stainsby (1987) that larger particles are often the source of emulsion instability. The peptide films were obviously not effective in preventing continued recoalescence or flocculation of the oil droplets, a phenomenon that started during formation of the emulsions (because of the bimodal shapes of the size distributions). So not only are the tryptic peptides of β-lg poor emulsifiers, they were also poor stabilizers, as found by Turgeon *et al.* (1992). Increasing the amount of hydrolyzates from 0.5 to 1% (Figure 6B), however, contributed to a better storage stability, possibly because the increased amount of native β-lg was able to stabilize more surface more effectively. Emulsions formed from 1% hydrolyzed β-lg were smaller in average size and more stable, which is in agreement with the fact that small particles are generally less susceptible to destabilization (Walstra, 1983; Dickinson and Stainsby, 1987).

The comparison of typical storage stability of the three sets of emulsions (nonhydrolyzed, solution-hydrolyzed, and surface-hydrolyzed) is also shown in Figure 6. This comparison highlights the instability of emulsions formed with hydrolyzed solutions compared with the surface-hydrolyzed or the unhydrolyzed emulsions. There was virtually no difference in the size distributions of the latter two emulsions. Thus, the hydrolysis of the adsorbed protein did not, of itself, cause destabilization. Moreover, the surface-hydrolyzed emulsions were as stable, if not slightly more stable, to storage than the unmodified emulsions. This result suggests that there were significant differences in the products of hydrolysis, or that different peptides are adsorbed to the interface, between proteins hydrolyzed before and after emulsion formation. It is especially noteworthy that surface-hydrolyzed emulsions containing β-lg are much more stable than their counterparts in which the protein was pre-hydrolyzed.

It is perhaps significant the hydrolysis of the emulsions has no effect on their ζ-potentials. We found no significant differences ($p \geq 0.05$) between nonhydrolyzed emulsions and surface-hydrolyzed emulsions, even after long hydrolysis times. However, we also found that emulsions formed with hydrolyzed proteins had comparable ζ-potentials with those formed with intact proteins. We have previously found (Dalgleish *et al.*, 1995) no significant differences between the ζ-potentials of emulsions formed with caseinate at concentrations ranging between 0.5 and 2%. These results suggest that there was no difference in the surface potential irrespective of the protein or polypeptide type, content, or structure. Although the significance of these results is not very clear, it suggests that in no cases were the emulsions stabilized predominantly by charge, because both stable and unstable emulsions had the same ζ-potential.

CONCLUSION

The comparison of the hydrolysis of caseinate and β-lg in aqueous solution as well as on the surface of an emulsion highlights the importance of protein structure and conformation in its functionality. The hydrolysis of milk protein solutions for effects of the proteins on emulsifying properties, especially at neutral pH, should be treated with caution because hydrolysis does not always lead to improvements. It is significant that hydrolysis of proteins adsorbed to the interface still produced very stable emulsions whose stability was even marginally improved over that of unhydrolyzed emulsions. In this study we have described the formation of emulsions involving hydrolyzed protein; the influence of changing environmental conditions on the stability of the emulsions containing hydrolyzed proteins will form a second part of this study.

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