Protease Stabilization by Highly Concentrated Anionic Surfactant Mixtures

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ABSTRACT: We have found that anionic surfactants such as linear alkylbenzene sulfonate (LAS) can solubilize proteases in a substantially nonaqueous environment without loss of proteolytic activity. Moreover, in mixtures of anionic and nonionic surfactants with a moderate amount of water (water less than 30 wt%), controlled levels of LAS and water solubilize proteases; yet, in these concentrated surfactant mixtures, enzymes maintain their activity for extended periods. Experimental design techniques have been used to delineate the relationship between protease stability and the water, pH and anionic surfactant levels in these surfactant concentrates. As the sum of water and LAS levels is increased, maximum enzyme stability is observed, after which stability falls off. At low water and LAS levels (sum of both <20%), protease solubility is low, while at high levels of water and LAS (sum of LAS and water >45%), denaturation predominates. Additionally, we have developed a new and simple method to predict protease stability by which a synthetic peptide is used to measure protease activity directly in the surfactant concentrate. From the application of this new technique to our system and to commercial liquid detergent formulations, it is apparent that water facilitates the loss of activity of proteases in surfactant concentrates by increasing the rate of autolysis.

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KEY WORDS: Alkylbenzene, anionic, concentrated, detergent, HDL, protease, stabilization, sulfonate, surfactant.

The formulation of enzymes in heavy-duty detergent liquids has been a challenge over the last thirty years. Enzymes are commonly added to detergent powders and liquids to improve the performance of these products on biological soils. The most commonly used detergent enzymes are the proteases, which break down proteinaceous soils such as blood and grass. It is well known that surfactants, especially anionic and cationic surfactants, interact strongly with enzymes (1–3). Surfactants interact with the protein, disrupting the tertiary structure of the enzyme (denaturation), resulting in the loss of activity and, in the case of protease enzymes, increasing self-digestion (autolysis). Moreover, anionic surfactants can complex and remove calcium ions, which act as structural cross-links across folds in the protein backbone of many bacterial proteases (4,5).

Recently, acceptable stabilization strategies for the incorporation of proteases into liquid detergents have been developed. The most effective stabilization schemes for aqueous detergent liquids (>50% water) are based on salts such as borate (6) and formate (7), which have been shown to act as protease inhibitors (8,9), thus slowing the rate of autolysis. It is unclear whether these salts also act to prevent denaturation of proteins. An alternative stabilization strategy is to prevent autolysis by formulating substantially nonaqueous detergent liquids, thus limiting the water activity of the matrix and thereby limiting autolysis. In recent years, over 100 patents have been issued that relate to the formulation of nonaqueous liquids, and almost all of these patents describe liquids based on a suspension of solids (bleach, enzyme, calcium sequestering agent, etc.) in an alcohol ethoxylate matrix (10). Detergent proteases are not soluble in nonaqueous mixtures of nonionic surfactants and thus are present as suspended solids. Enzymes show excellent stability in nonionic surfactant mixtures, but because these proteins are not soluble, such mixtures are typically kinetically unstable dispersions. Formulations based solely on nonionic surfactants suffer in performance, especially on particulate soils.

Over the last ten years, there has been much interest in the use of enzymes in organic solvents. It has been shown by Klibanov (11) and Wong (12) that enzymes can function in substantially nonaqueous organic solvent systems, often with remarkable stability and altered reactivity. Enzymes, however, are not generally soluble in most polar aprotic solvents and are thought to act as suspended particles with their associated aqueous micro-environment. Stripping away this sphere of hydration, either by desiccants or polar solvents, results in denaturation of the enzyme and loss of catalytic activity (13). In highly polar solvents, such as dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF), many enzymes show some finite solubility. However, they are most often rapidly denatured in this unnatural environment. This denaturation is not surprising because the active conformation of the enzyme catalyst results from the delicate balance of intramolecular forces between amino acid residues and intermolecular interactions between the polypeptide chain and water. This delicate balance of a multitude of forces is disrupted by re-

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placing the aqueous environment with a polar organic solvent.

From what is known about the interaction of enzymes with organic solvents, the dissolution of a protease into true solution in a substantially nonaqueous mixture of anionic surfactants should result in rapid and complete denaturation of the protease. However, in one report (14), substantially nonaqueous surfactant system based on an anionic surfactant, linear alkylbenzene sulfonate (LAS), and an ethoxylated fatty alcohol (AE) was found to be a stabilizing media for proteases. From the report, it is unclear whether the protease was present as suspended particulates or dissolved in true solution. We decided to investigate this anomalous finding further.

Our paper describes a unique enzyme stabilization strategy, one based on the solubilization of proteases by anionic surfactant. We have found bacterial proteases to be soluble, yet remarkably stable in a substantially nonaqueous matrix of anionic and nonionic surfactants. Additionally, in a low-water formulation, in which the water content is balanced against the level of anionic surfactant, we have found that the anionic surfactant serves to dissolve the bacterial protease, yet these enzymes are stable for prolonged periods.

EXPERIMENTAL PROCEDURES

Materials. LAS, with an average carbon chainlength of 11.4 (SA® 597), and AE, based on $C_{12,14}$ linear Ziegler alcohol condensed with 60 wt% ethylene oxide (ALFONIC® 1412-60), are products of Vista Chemical Company (Houston, TX). Protease enzyme concentrate was a gift from Novo Nordisk Bioindustrials (Danbury, CT). Monoethanolamine (MEA) and triethanolamine (TEA) were obtained from Texaco Chemical Co. (Austin, TX). Azocasein and succinyl-L-alanyl-L-alanyl-L-prolinyl-L-phenylalanyl-paranitroanilide (sAAPFpNA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other materials were of reagent grade and were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Stability and solubility tests. A 4% (vol/vol) solution or suspension of the liquid protease concentrate Savinase® (Novo) 8.0L was prepared in each solvent system. The solution (or suspension) was allowed to stand for 1 h at 23°C, and then the proteolytic activity was assayed in 0.25M, pH 8.5 buffer by the azocasein assay (see below). The mixtures were then centrifuged for 10 min at 14,000 rpm (Eppendorf 5415C Microcentrifuge, Westbury, NY), and the activity of the clear solution (or supernatant in the case of a suspension) was determined.

Formulation of the surfactant concentrates. The formulations were prepared by adding the appropriate amount of linear alkylbenzene sulfonic acid (SA® 597) to the AE, which was based on C_{12-14} linear fatty alcohol condensed with 7 moles of ethylene oxide (ALFONIC® 1412-60). Alkanolamine was added sufficient to neutralize the sulfonic acid. To this mixture was added propylene glycol, calcium chloride and water, and the "pH" of the formulation was adjusted to the appropriate level by the addition on alkanolamine. The formulation "pH" in this low-water mixture is defined as the pH resulting from a solution of 1.5 g/L of the formulation in distilled water. After the pH of the formulation was adjusted, the protease concentrate (Savinase® 8.0L) was added at a level of 4% (vol/vol) to the formulation. The protease activity of the resulting formulation was measured by the azocasein assay. The 100% enzyme activity level corresponds to the activity of the enzyme prior to addition to the surfactant concentrate. Samples were incubated in a temperature-controlled waterbath at 40°C, and the protease activity was determined at regular intervals. The formulation compositions are listed in the Results and Discussion section. In the central composite experimental design, the run order was randomized, and the experiment was split into two blocks. Stability studies for the two blocks were initiated on consecutive days.

Protease assays. Throughout the stability studies, 10-μL aliquots were removed periodically, and the protease activity was determined with azocasein as a substrate in a manner similar to that previously described (15). In an optimized procedure, the azocasein substrate was first prepared by dissolving 0.60 g of azocasein (Sigma Chemical Co.) in 10 mL of 50% urea (wt/vol), taking care to completely dissolve the azocasein. To this solution was added 10 mL of 2 M Tris (pH 8.5 unless indicated otherwise), which contained 500 ppm of $CaCl_2$. The volume of the azocasein solution was adjusted to 100 mL with deionized water. A 10-μL aliquot of the protease/surfactant concentrate was dissolved in 1 mL of 0.2 M Tris buffer (pH 8.5) containing 50 ppm of $CaCl_2$. The assay solutions were preheated for 1 min at 40°C, then 5 mL of the azocasein solution, which had been preheated to 40°C, was added with mixing. The tubes were incubated at 40°C, and the digestion was stopped after exactly 30 min by the addition of 5 mL of a 5% trichloroacetic acid solution. After standing for 20 min at room temperature, the precipitated azocasein was removed by centrifugation at 14, 000 rpm (Eppendorf® 5415), and the absorbance of the supernatant was measured at 390 nm. Each sample was run in duplicate, and an average is reported here. A blank determination, containing all but the protease, was also run, and the absorbance of the blank was subtracted from that of the protease samples. A sample containing nonsurfactant-treated protease was also assayed, and the result was used as the 100% protease activity level.

To determine the proteolytic activity in the actual detergent liquid, the substrate sAAPFpNA (16) (50 μL of a 10-mM DMSO solution) was added directly to 1.5 mL of the protease-containing detergent liquid. The sample was incubated at 25°C, and the rate of release of paranitroaniline was monitored at 410 nm. Absorbance measurements were made with a Hewlett-Packard 8452A photodiode array spectrophotometer (Palo Alto, CA).

Ultrafiltration. Ultrafiltration of the formulations was performed in a 70-mL stirred cell fitted with a 50,000 MW cutoff cellulose acetate membrane (Cole-Parmer, Niles, IL) at a

pressure of 50 psi. The retentate and the permeate were analyzed for enzyme activity by the azocasein assay.

RESULTS AND DISCUSSION

Protease solubility and activity in nonaqueous systems. The solubility of the bacterial protease was tested in a number of solvent systems, and the results are shown in Table 1. It is apparent that the solubility of the protease is negligible in DMF or in the alcohol ethoxylate. In DMSO, the enzyme is soluble, but it is completely denatured in the dissolution process. This is to be expected because the delicate balance of intramolecular interactions that lead to the active conformation of the enzyme catalyst by necessity is disrupted by replacing the aqueous environment with an organic solvent. During the precipitation from DMF, the protease loses most of its activity. The AE does not dissolve the protease, but during the precipitation process, no enzyme activity is lost. This is consistent with the wide body of literature describing the long-term stability of enzymes suspended in a nonaqueous AE matrix. Surprisingly, the anionic/nonionic and the purely anionic surfactant systems act as solvents for the protease but do not denature it.

To test the homogeneity of the anionic surfactant-based protease solution, the LAS/AE system was filtered through an ultrafiltration membrane with a 50,000 dalton cutoff. The activity of the retentate did not change during the filtration, while a full 84% of the protease activity passed through the membrane (the rest was most likely lost to adsorption on the filter), indicating that the enzyme is present as a unimolecular species in true solution, or at least in equilibrium with a unimolecular species. It appears that the anionic/nonionic blend acts as a solvent for the enzyme but does not denature it. Unexpectedly, the anionic surfactant here is the agent that solubilizes the protein while still maintaining complete activity of the protein catalyst.

One implication of the finding that nonaqueous anionic surfactant concentrates dissolve but do not denature proteases is that these systems can be used for the formulation of enzyme-stable, highly concentrated heavy-duty detergent liquids. In these formulations, the anionic surfactant acts as a formulation aid for the enzyme, allowing one to substantially lower the water level of a detergent liquid while maintaining an isotropic solution of the enzyme with excellent stability. The incorporation of an anionic surfactant also greatly improves the performance of the detergent liquid, especially on particulate soil.

Optimization of protease stability in anionic surfactant concentrates. It was evident to us from the large body of literature on the stabilization of proteases in detergent liquids, that there are quite a large number of factors that affect protease stability (1,17,18). It is well known, for example, that, in the absence of stabilization agents, anionic surfactants in water-based detergent liquids have a negative effect on enzyme stability while nonionic surfactants typically do not denature proteins. From our results in the solubilization of proteases in anionic surfactant concentrates (above), combined with the knowledge that proteases are stable in aqueous and nonaqueous nonionic surfactant mixtures, we realized that one could prepare enzyme-stable formulations based on a wide range of surfactant and water mixtures by limiting the "free" water. In our estimation, the variables likely to be important in formulating a protease/surfactant concentrate mixture included wt% water, anionic surfactant, counter-ion identity, propylene glycol and formulation pH. The delineation of the relative importance of a large number of factors such as those listed here is most efficiently performed, by using experimental design techniques (19,20). A Plackett-Burman (21) screen was used to determine which of the above factors had the greatest impact on protease stability. An estimate of the relative importance of a factor is obtained simply by the addition of a response factor for each experiment, when that factor is at a high level, and the subtraction of the experimental response, when the factor in question is at a low level (22). The design is outlined in Table 2.

From analysis of the results (Table 3), it is apparent that the major factor in determining enzyme stability is not a single factor but the interaction of three factors: water, LAS and pH. The effect of each factor alone is an order of magnitude lower than the three-way interaction.

TABLE 1
Protease Solubility and Stability in Aqueous and Organic Solvent Systems

Solvent[a]	Precipitate	% Activity in supernatant	% Activity of precipitate
Tris buffer, pH 8.5	No	100	NA
DMSO	No	0	NA
DMF	Yes	0.3	15.8
LAS–TEA/alcohol ethoxylate (50:50)	No	99.7	NA
LAS-TEA/propylene glycol (95:5)	No	95.3	NA
Alcohol ethoxylate	Yes	0	98.7

[a]4% (vol/vol) Savinase® 8.0L (Novo Nordisk, Danbury, CT) was added to each solvent mixture. The resulting solutions contain 1.5% water. DMSO, dimethyl sulfoxide; DMF, dimethyl formamide; LAS–TEA, linear alkylbenzene sulfonate–triethanolamine. NA, not applicable.

TABLE 2
Design Table for Plackett-Burman Screening Design: Protease Stability in a Concentrated Surfactant Mixture[a]

	Coded level of variable	
Factor	-1	+1
	Actual level of variable	
pH (X_1)	7	9.5
Water (X_2)	1.5%	15%
Propylene glycol (X_3)	0%	10%
LAS (X_4)	15%	35%
Counter-ion (X_5)	MEA	TEA

[a]The balance of each formulation is alcohol ethoxylate. The experimental response is percent of remaining protease activity on storage at 40°C. MEA, monoethanolamine. See Table 1 for other abbreviations.

TABLE 3
Percent Remaining Protease Activity After 52 Days of Storage At 40°C[a]

Experiment	pH	Water (wt%)	Proplylene glycol (wt%)	LAS (wt%)	Counter-ion	% Activity day 52
1	7.0	1.5	0	15	MEA	48.1
2	7.0	1.5	0	35	TEA	29.2
3	7.0	15	10	35	TEA	73.2
4	7.0	15	10	15	MEA	31.2
5	9.5	15	0	15	TEA	81.4
6	9.5	15	0	35	MEA	14.6
7	9.5	1.5	10	35	MEA	29.6
8	9.5	1.5	10	15	TEA	0.5

[a]See Tables 1 and 2 for abbreviations.

While the AE level is widely varied in the above experiments, the effect of this variable is not treated separately. We have relied on previous studies, which have shown the excellent stability of proteases in aqueous, nonionic formulations and the insolubility of the protein in nonaqueous nonionic formulations. If this assumption is incorrect, our results are convoluted by the variation of AE. Some qualitative observations can be made about the AE level. We observed that precipitate formation accounted for the loss of enzyme activity in experiments 1 and 8. While the effects of AE are not separated out in these experiments from the results of the solubility experiments (Table 1) it is likely that the low-water and anionic surfactant levels resulted in the precipitation of the protease. Thus, solubility rather than denaturation accounts for the loss in enzyme activity in experiments 1 and 8. Experiment 4, having a high level of AE, a low level of LAS and a high level of water, shows intermediate protease stability.

Remarkably, experiments 3 and 5, having relatively high levels of both water and LAS, show superior protease stability. This result runs contrary to what is known about the stability of proteases in anionic surfactant mixtures. Substantially nonaqueous formulations 1, 2, 7 and 8 unexpectedly showed less enzyme activity than the higher water and LAS content formulations 3 and 5, most likely as a result of the higher solubility of protein in these mixtures. This unexpected finding was investigated further.

To better map out the effect of the water/LAS/pH interaction and to determine where the optimal stability region is located, a 2^3 full-factorial experimental design was performed. The water content was varied from 1.5 to 30%, the formulation pH from 7 to 9.5, and LAS from 15 to 35% (results not shown here). Unexpectedly, the stability of the center point of this design (i.e., LAS = 25%, pH = 8.25 and water = 15.8) was higher than any other of the experimental points. This indicated that the relationship between enzyme stability and pH, water content and LAS content was not linear and that no useful model could be derived from this set of experiments.

To map out the region of maximum enzyme stability, a central composite experimental design (CCD) (23) was performed with three variables: pH (X_1), water (X_2) and LAS (X_3). A central composite design allows one to derive higher-order polynomial relationships and to generate a response surface that describes enzyme stability over a range of effects. The computer program "Design-Expert" (24) was used to set up the experiment, analyze the data, and to calculate a mathematical relationship that best describes the relationship between enzyme stability and the experimental variables. The CCD included 8 factorial points with repetition of 4 of the points, 6 center point replicates and 6 axial points in duplicate. Table 4 lists the coded and actual variable levels, while the results of the series of experiments are reported in Table 5.

A nonlinear relationship was found between enzyme stability and the factors LAS, water and pH. The results of the

TABLE 4
Coded and Actual Levels for the Variables pH, Water and LAS for the Central Composite Design[a]

	Coded level of variable				
	-1.68	-1	0	1	1.68
Variable	Actual level of variable				
pH (X_1)	7.0	7.5	8.25	9.0	9.5
Water (X_2)	1.5	7.0	15.0	23.0	28.5
LAS (X_3)	15.0	19.0	25.0	31.0	35.1

[a]See Table 1 for abbreviation.

TABLE 5
Percent Remaining Protease Activity After Storage at 40°C for 45 Days[a]

pH	% Water	% LAS	% Activity
9.0	23.0	19.0	63
9.0	7.0	31.0	64
7.5	7.0	31.0	58
8.25	15.0	25.0	54
7.5	23.0	31.0	25
7.5	7.0	19.0	35
9.0	23.0	31.0	42
8.25	15.0	25.0	58
7.5	23.0	19.0	28
8.25	15.0	25.0	58
8.25	15.0	25.0	58
9.0	7.0	19.0	55
8.25	15.0	35.0	47
9.5	15.0	25.0	67
7.0	15.0	25.0	35
8.25	28.5	25.0	42
8.25	15.0	25.0	60
8.25	15.0	25.0	58
8.25	15.0	15.0	53
8.25	1.5	25.0	55
7.5	23.0	31.0	34
9.0	7.0	19.0	64
7.5	7.0	31.0	65
7.0	15.0	25.0	33
9.5	15.0	25.0	61
8.25	15.0	15.0	52
8.25	15.0	35.0	47
8.25	28.5	25.0	34
7.5	7.0	19.0	35
8.25	1.5	25.0	55

[a]See Table 1 for abbreviation.

CCD experiment were fitted to a second-order polynomial equation:

$$Y = 60.06 + 9.28X_1 - 6.1X_2 - 0.33X_3 - 3.16X_1^2 - 4.13X_2^2 - 2.88X_3^2 + 2.88X_1X_2 - 6.05X_1X_3 - 6.80X_2X_3 \quad [1]$$

Enzyme stability (Y) increased with pH (X_1) and decreased with water (X_2) and LAS (X_3). The cumulative result is more apparent from the contour plots of the response surface (Figs. 1–4).

The derived model fits the data well, as seen from the analysis of variance (Table 6). The F value is the ratio of the mean square variation from the selected model to the mean square variation derived from experimental error (estimated from repetition of the factor space center point. The large F value indicates that the variance explained by the model is much greater than that which would be expected from random variation and that the model is statistically significant. The closeness of the correlation coefficient (R^2) to unity indicates that the quadratic model accounts for 94% of the variability. The lack-of-fit test (Table 7) shows that there is little probability that the quadratic model is not significant and that the quadratic model explains the results much better than does a linear model. In the lack-of-fit test, the low F value indicates a low probability of a lack of fit for the quadratic model, i.e., the probability that the quadratic model fits is high.

The contour plots of percent of remaining activity for a

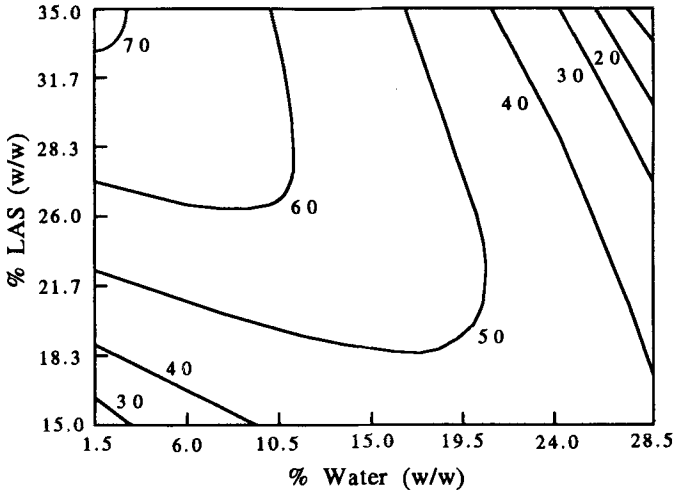

FIG. 1. Percent remaining protease activity on storage at 40°C in concentrated linear alkylbenzene sulfonate (LAS)/ethoxylated fatty alcohol mixtures at pH 8.0.

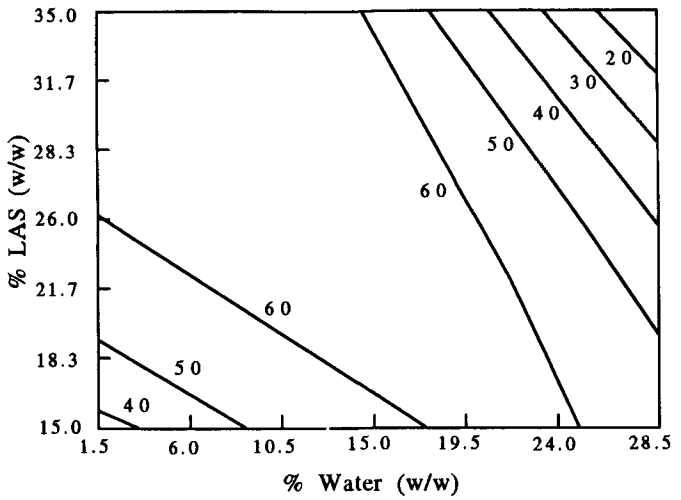

FIG. 2. Percent remaining protease activity on storage at 40°C in concentrated LAS/ethoxylated fatty alcohol mixtures at pH 8.5. See Figure 1 for abbreviation.

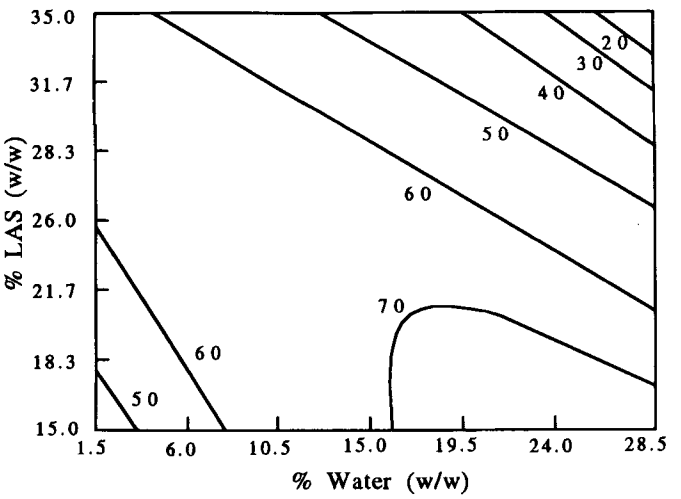

FIG. 3. Percent remaining protease activity on storage at 40°C in concentrated LAS/ethoxylated fatty alcohol mixtures at pH 9.0. See Figure 1 for abbreviation.

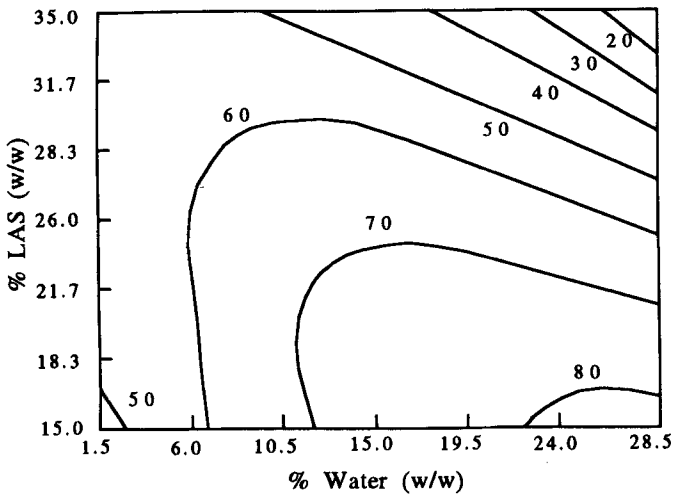

FIG. 4. Percent remaining protease activity on storage at 40°C in concentrated LAS/ethoxylated fatty alcohol mixtures at pH 9.5. See Figure 1 for abbreviation.

TABLE 6
Analysis of Variance: Summary of Fit for Quadratic Model

Source of variation	DF[a]	Sum of squares	Mean square	F Value
Model	9	4478.4	497.6	36.9[b]
Error	20	269.6	13.5	
Total	29	4748.0		
Root mean square error = 3.67				
$R^2 = 0.943$[c]				

[a]Degrees of freedom.
[b]Significant at the 1% confidence level.
[c]Multiple correlation coefficient.

TABLE 7
Lack-of-Fit Test

Model	DF[a]	Sum of squares	Mean square	F value
Linear	11	1665.98	151.45	12.58
Quadratic	5	88.95	17.79	1.48
Pure Error	15	180.62	12.04	

[a]Degrees of freedom.

TABLE 8
Comparison of Relative Rates of Enzymatic Peptide Hydrolysis and Long-Term Enzyme Stability in Concentrated Surfactant Mixtures

Formulation (LAS/AE/water)	Initial rate of *p*NA release Δabsorbance at 410 nm/min)	Half-life on storage (days at 40°C)
1. 40/42/0.5[a]	1.58×10^{-3}	33
2. Z5/42/15[a]	9.92×10^{-5}	47
3. 19/32/35[a]	2.34×10^{-1}	0.6
4. Model HDL without borax[b]	1.44×10^{-2}	2.5
5. Model HDL with borax[b]	1.70×10^{-3}	11

[a]Contains 10% propylene glycol, 1% SAVINASE® 8.0L and 500 ppm $CaCl_2$; pH adjusted to 9 with MEA.
[b]From Reference 25: 9% sodium LAS, 6.5% sodium alcohol ether sulfate, 7% ethoxylated alcohol (AE), 7% sodium citrate, 5% sodium xylene sulfonate, 2% TEA, 2% MEA, 500 ppm $CaCl_2$, 1% SAVINASE® 8.0L, water to 100%. HDL, high-density lipoprotein. See Tables 1 and 2 for other abbreviations and source information.

range of LAS and water levels are shown in Figures 1–4. The model indicates that, over the range of concentrations studied the LAS and water levels must be balanced against each other to maintain enzyme stability. A band of optimal stability is found along the diagonal where as the LAS level is increased, and the water content is lowered. Apparently, it is the sum total of the water and LAS levels, rather than either one taken alone, that is important. At low levels of LAS and water, solubility of the enzyme is low, and precipitation is observed. This precipitate contains virtually all of the enzyme activity, so denaturation does not occur in these low-water, high-AE mixtures. At high levels of LAS and water, solubility is good, but stability is poor.

We believe that the mechanism for the loss of enzyme activity at high LAS and water levels is a combination of denaturation and autolysis. This model has been used by Crossin to explain the stabilization of proteases in high-density lipoproteins (HDLs) by short, chain carboxylic acids. In our system, it is probable that excessive "free" water results in an increase of autolysis, while excessive LAS aids in autolysis by denaturation of the protein structure.

To further elucidate the mechanism of the loss of enzyme activity, the protease activity was determined in the actual detergent liquid. The protease substrate sAAPFpNA was added directly to stabilized and unstabilized detergent formulations and to the concentrated surfactant mixtures of this study. This technique differs from others in that the activity of the enzyme is measured "in the bottle," rather than looking at activity indirectly or through model systems.

From Table 8, it is obvious that the proteolytic activity in the actual formulation is a good (and much less time-consuming) predictor of the ultimate stability of the protease in the formulation. There is a linear relationship between the logarithm of the initial rate of peptide hydrolysis and the ultimate storage stability of the protease in the product. The excellent correlation of *in situ* peptide hydrolysis activity with long-term protease stability implies that for these formulations, autolysis is responsible for a large part of the loss of enzyme activity, rather than unimolecular denaturation. In the optimized low-water formulations (1 and 2 in Table 8), the "in the bottle" activity is two or three orders of magnitude lower than in the higher 2 water formulations. Formulation 3, which has a much higher activity "in the bottle," has a half-life of less than one day. This same predictive technique, measurement of the proteolytic activity of a formulation was applied to aqueous HDLs. Borate in the presence of a polyol is known to provide excellent protease stabilization in detergent liquids (25). Formulation 4 with no borax had 4.4 times less stability than formulation 5, which contains borax. This implies that, at least to some degree, the mode of stabilization in both the low-water anionic-based formulations and the borax-stabilized aqueous formulations is by inhibition of autolysis.

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