Development of Off-Flavors in Ultra-High Temperature and Pasteurized Milk as a Function of Proteolysis[1]

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ABSTRACT

The relationship between proteolysis and off-flavor development in ultra-high temperature and pasteurized milk was investigated. Milks were subjected to proteolysis by different concentrations of three psychrotroph enzymes for 20 h at 35°C. Proteolysis, measured as the increase in trichloroacetic acid-soluble free amino groups was subsequently determined colorimetrically with trinitrobenzene sulfonic acid. Bitter off-flavors were determined by a 10-membered panel.

Background trichloroacetic acid-soluble free amino groups of .819, .822, and .817 μmoles/ml were determined for raw, pasteurized, and ultra-high temperature milk, respectively. Increases in free amino groups were significant when pasteurized milk was incubated at both 4°C (.87) and 35°C (.925) for 20 h.

Ultra-high temperature milk was approximately twice as sensitive as pasteurized milk to the action of crude proteolytic enzymes, but unlike pasteurized milk, it did not coagulate when exposed to high concentrations of enzyme.

When increasing volumes of crude proteases were added to ultra-high temperature and pasteurized milk, proteolysis could be detected in samples which had not developed off-flavors. Off-flavors were detected in ultra-high temperature milk at proteolysis (change μmoles/ml) of .554, .355, and .287 for the three proteases. Corresponding values for pasteurized milk were .499, .355, and .746.

Electrophoretic profiles of caseins isolated from protease-treated ultra-high temperature and pasteurized milk did not differ markedly from those of untreated milk. In all samples receiving enzyme treatment a slow moving band was detected that increased in intensity with increased enzyme concentration. Detection of low levels of proteolysis with trinitrobenzenesulfonic acid might have some application in monitoring stored ultra-high temperature milk.

INTRODUCTION

The current practice of storing raw milk at 4 to 7°C for up to 4 days prior to processing permits growth of psychrotrophs (9). These microorganisms produce heat-stable proteolytic enzymes (1, 11) that survive ultra-high temperature (UHT) processing and cause gelation and the development of bitterness in the finished product (10, 13, 19).

Law (9) and Richardson and Newstead (13) have pointed out the need for sensitive methods of determining trace amounts of proteases in milk. Proteolysis in milk is often measured as the release of acid-soluble tyrosine by the method of Hull (5). This method is somewhat insensitive since proteolytic breakdown products of milk proteins frequently lack tyrosine residues (12). The Hull method has been used successfully to show a correlation between proteolysis and off-flavor in milk (7, 11, 18) but was unable to detect proteolysis in unspoiled milk. Results were similar when proteolysis was measured as the increase in nonprotein nitrogen (13).

Cogan (2) suggested that proteolysis of milk could be measured with nonspecific amino reagents such as ninhydrin or trinitroben-

zenesulfonic acid (TNBS). Schewedtfeger (16) tested several protease assays and found that the TNBS method was especially good for milk. My report shows the relationship between proteolysis measured with TNBS and off-flavor development.

MATERIALS AND METHODS

Strains and Culture Conditions

Strains for this study were isolated from raw milk obtained from the Central Experimental Farm in Ottawa. They were assigned numbers 13, 15, and 33 and maintained by monthly transfer onto nutrient agar (Difco) slants at 7°C.

Identification of Strains

Preliminary identification was by gram reaction, motility, oxidase reaction, and presence or absence of fluorescent pigment. Microorganisms were further classified by the API 20E system (API Laboratory Products Ltd., St. Laurent, Que.) according to manufacture's specifications with the exception that incubation was at 22°C. The ability of the isolates to utilize various carbon sources was tested by the disc method of Rosenthal (14). Carbon sources were arabinose, sorbitol, propionic acid, dextrose, sucrose, saccharic acid, adonitol, inositol, melibiose, trehalose, absolute ethanol, propylene glycol, and butyric acid. Growth was measured after incubation for 48 h at 22°C.

Preparation of Cell-Free Enzyme

Fifty milliliter volumes of nutrient broth containing .2% reconstituted skim milk in 250-ml flasks were inoculated with 1% of an overnight culture and incubated at 22°C for 24 h with shaking at 150 rpm. Cells were removed by centrifugation at 12,000 × g for 10 min, and supernatants were filter-sterilized (Millipore, .45μ). The cell-free enzyme preparations were stored in 1-ml lots at −80°C until required.

Enzyme Treatment of Milk

The UHT milk (2% milk fat) produced by Cité Dairies, Quebec City, was obtained from a local supermarket in 1-liter Tetra Brik cartons. Milk was from a single batch and was stored at 25°C until used. Raw milk, obtained daily from the Central Experimental Farm in Ottawa, was the product of two milkings and had been stored at 4°C for not more than 18 h. Raw milk was centrifuged at 480 × g for 10 min, and cream removed by suction. Three liters of skim milk in a 4-liter Erlenmeyer flask were pasteurized in a 62°C waterbath for 30 min. Neomycin sulfate was added (10 μg/ml) to suppress bacterial growth, and milk was stored at 4°C.

One hundred and fifty milliliter of milk were

TABLE 1. Biochemical characteristics of *Pseudomonas fluorescens* strains 13, 15, and 33.

Test	Strain		
	13	15	33
Fluorescent[a] pigment	−	−	+
ADH[b]	+[d]	−	+
GEL	+	+	+
CIT	+	+	+
NIT	−	−	+
GLU	+	+	+
SAC	−	−	−
MEL	−	−	+
ARA	+	+	+
Arabinose[c]	+	+	+
Sorbitol	+	+	+
Propionic acid	+	+	+
Dextrose	+	+	+
Sucrose	−	+	−
Saccharic acid	+	+	+
Adonitol	±	±	±
Inositol	+	+	+
Melibiose	−	−	−
Trehalose	+	+	+
Ethanol	−	−	±
Propylene glycol	−	−	±
Butyric acid	−	−	−

[a]Fluorescent pigment was detected on nutrient agar containing 1% reconstituted skim milk after incubation at 22°C for 48 h: +, pigment produced; −, pigment not produced.

[b]Tests were from API 20E; ADH, arginine dihydrolase; GEL, gelatin hydrolysis; CIT, utilization of citrate; GLU, acid produced from glucose; SAC, acid produced from saccharose; MEL, acid produced from melibiose; ARA, acid produced from arabinose.

[c]Tests were based on the ability to use the indicated compound as the sole source of carbon.

[d]Growth was determined after incubation at 22°C for 48 h; +, growth; −, no growth; ±, slight growth.

added aseptically to sterile 250-ml Erlenmeyer flasks. Various concentrations of cell-free cultural filtrate from one of the three strains were added to duplicate flasks and shaken at 100 rpm at 35°C for 20 h. Duplicate controls lacking enzyme were also shaken at 4 and 35°C. After the incubation period, 2-ml samples from each duplicate flask were kept for analysis of free amino groups. Flasks then were cooled in ice for 2 to 3 h and warmed to 18 to 20°C. Duplicate flasks were combined and submitted to sensory evaluation. Samples of milk also were stored at −80°C for analysis by polyacrylamide disc gel electrophoresis.

Each experiment utilizing culture filtrate from a single strain and either UHT or pasteurized milk was repeated on three occasions, and values for off-flavor and proteolysis were averaged.

Sensory Evaluation

Ten experienced judges evaluated the milk samples for off-flavor by the method of descriptive analysis with scaling (8). Each sample was compared to the 4°C control and rated for intensity of off-flavor on a 15-cm linear scale ranging from no difference to extreme difference. In addition, judges were asked to describe off-flavor. The mean off-flavor scores for the three replicates of each treatment sample were calculated. Significant differences between means for the 35°C controls and those for samples containing enzyme were determined by analysis of variance. When differences were detected, they were calculated to 5% probability by Tukey's test.

Analysis of Free Amino Groups

Free amino groups were determined by the method of Fields (4) as modifed by Spadaro et al. (17). Duplicate 2-ml samples of milk were treated with 4 ml of .72N trichloroacetic acid (TCA) for 20 min at 25°C then filtered through Whatman #1 filter paper. Duplicate .2-ml volumes of each supernatant were mixed with 2 ml of 1 M potassium borate buffer (pH 9.2) and .8 ml of 5 mM TNBS (Sigma) and incubated in the dark at 25°C. Duplicate samples of standard solutions of glycine that had been treated with TCA but not filtered were also included.

After 30 min, .8 ml of 2 M monobasic sodium phosphate containing 18 mM sodium sulfite was added, and the absorbance at 420 nm was measured in a Beckman DB-G spectrophotometer. Absorbances were converted to micromoles of free amino groups per milliliter of milk by a standard curve. Proteolysis was defined as the increase in the concentration of trichloroacetic acid-soluble free amino groups per milliliter of milk (Δ μmoles/ml).

Polyacrylamide Disc Gel Electrophoresis

Casein fractions were isolated from UHT and pasteurized milk by the method of Richardson and Newstead (13). One milliliter samples of milk were diluted with 1.3 ml of water and mixed with .1 ml each of 10% acetic acid and 1 M sodium acetate. After incubation at 35°C for 10 min, the precipitate was collected by centrifugation at 3,000 × g for 10 min at 25°C, washed once with 2 ml of water, and resuspended in 1 ml of .02 N sodium hydroxide.

TABLE 2. TCA-soluble free amino groups in untreated milk.

Milk	Incubation conditions	Free amino groups[1] (μmoles/ml)	SE
UHT	. . . .	.817 (8)[ab]	.014
Raw	. . . .	.819 (17)[a]	.020
Pasteurized	. . . .	.822 (17)[a]	.017
Pasteurized	20 h, 4° C	.870 (10)[b]	.013
Pasteurized	20 h, 35° C	.925 (10)[c]	.009
UHT	20 h, 35° C	.810 (8)[ab]	.018

[a,b,c,d] Any two means not followed by the same letter were different ($P<.05$).

[1] Figures in brackets represent the number of replicates. Replicates for UHT milk were from the same batch of milk performed on separate days. Raw and pasteurized milk replicates were performed daily with fresh milk.

One milliliter of 40% sucrose was added, then urea and 2-mercaptoethanol (2-ME) were added to 7 M and 20 mM, respectively. Ten microliters of sample or casein standards, which had been treated with urea and 2-ME, were layered on top of each gel.

Electrophoresis was according to the method of Davis (3) with the exception that running gels contained 4.5 M urea and 20 mM 2-ME. Gels were fixed in 12.5% TCA, stained with 1.25% Coomassie brilliant blue (Bio-Rad Laboratories) and destained with 5% acetic acid.

RESULTS

The three strains in this study were gram negative, oxidase positive, and motile. Further biochemical characteristics are in Table 1. Strains 13 and 15 were similar biochemically; they could be distinguished only on the basis of their ability to utilize sucrose. These two strains did differ, however, with respect to the amount produced as well as heat stability of their proteases (R. C. McKellar, unpublished). All three organisms were judged to be strains of *Pseudomonas fluorescens*.

The levels of TCA-soluble free amino groups in untreated milk are in Table 2. Heat treatment of raw milk had little effect on TCA-soluble free amino groups; means for fresh raw and pasteurized milk were not significantly different from that of UHT milk. Incubation of UHT milk for 20 h at 35°C did not result in any significant increase in value over that obtained

for milk stored at 4°C. Amounts of free amino groups increased when pasteurized milk was kept for 20 h at 4°C. A more significant increase
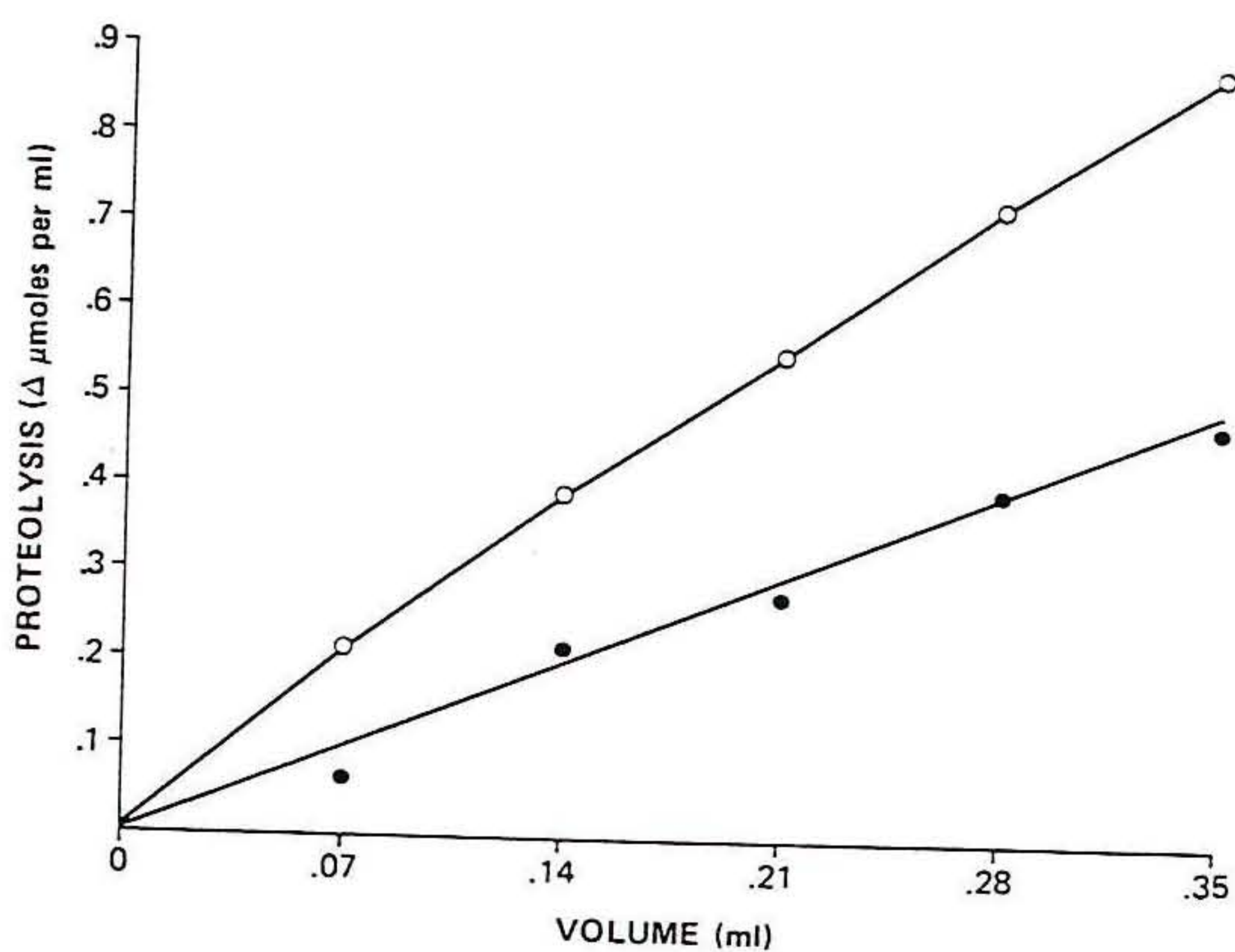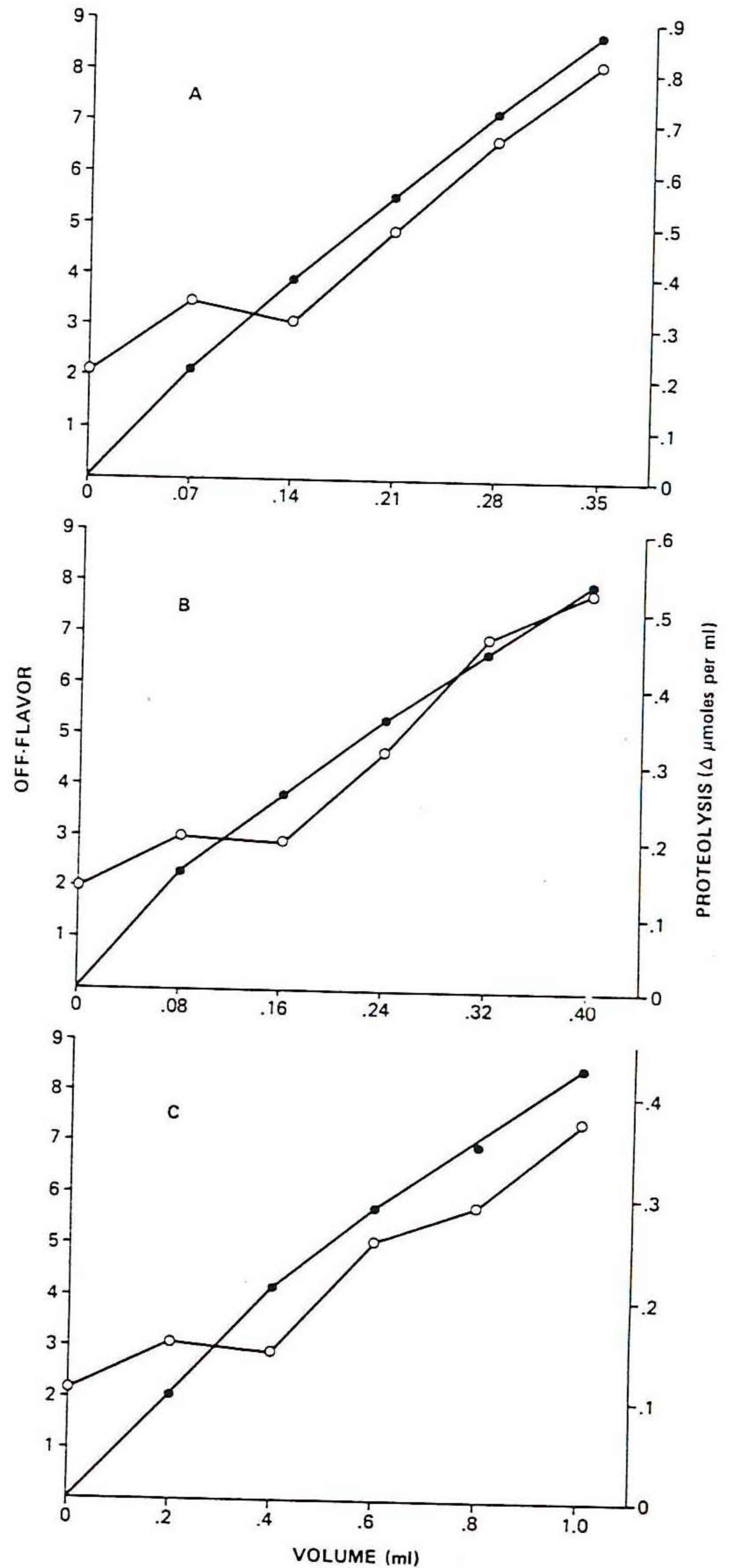

Figure 2. Effect of increasing volumes of cultural filtrate on proteolysis and intensity of off-flavor in UHT milk. a, strain 13; b, strain 15; c, strain 33. Off-flavor, o—o; proteolysis (Δ μmoles/ml), ●—●. Proteolysis was determined relative to the control at 35°C. For off-flavor and proteolysis, means were of three determinations. Standard errors of each mean for off-flavor and proteolysis were for enzyme 13, .336 and .024; for enzyme 15, .336 and .014; and for enzyme 33, .401 and .009.

Figure 1. Effect of increasing volumes of cultural filtrate from strain 13 on proteolysis in UHT and pasteurized milk. UHT, o—o; pasteurized, ●—●.

was observed when incubation was at 35°C (Table 2).

The effect of increasing volumes of cultural filtrate from strain 13 on proteolysis in UHT and pasteurized milk is in Figure 1. The enzyme was approximately twice as active against UHT as against pasteurized milk. Results were similar when cultural filtrates from strains 15 and 33 were used (data not shown).

Figure 2a shows the effect of increasing volumes of cultural filtrate from strain 13 on proteolysis and intensity of bitter or astringent off-flavors in UHT milk. Up to .14 ml of cultural filtrate could be added to 150 ml of milk with no significant development of off-flavor. Once off-flavors developed, the intensity increased in proportion to proteolysis.

Results were similar with cultural filtrates from strains 15 and 33 (Figure 2b, c). With these two strains, up to .16 and .4 ml of cultural filtrate could be added for strain 15 and 33, respectively, before off-flavors became significant.

Figure 3a shows the effect of increasing volumes of cultural filtrate from strain 13 on proteolysis and the intensity of bitter or astringent off-flavor in pasteurized milk. The proteolysis curve was concave, indicating that proteolysis did not increase in a strictly linear fashion with increasing volumes of cultural filtrate. Under similar conditions the proteolysis curve for UHT milk was convex (Figure 2a). Although off-flavor scores for pasteurized milk were generally lower than those for UHT milk, proteolysis was marked prior to the development of significant off-flavor (Figure 3a) as for UHT milk (Figure 2a).

Results were essentially the same with cultural filtrates from strains 15 and 33 (Figure 3b, c). With these two enzymes, large increases in off-flavor scores were observed with the
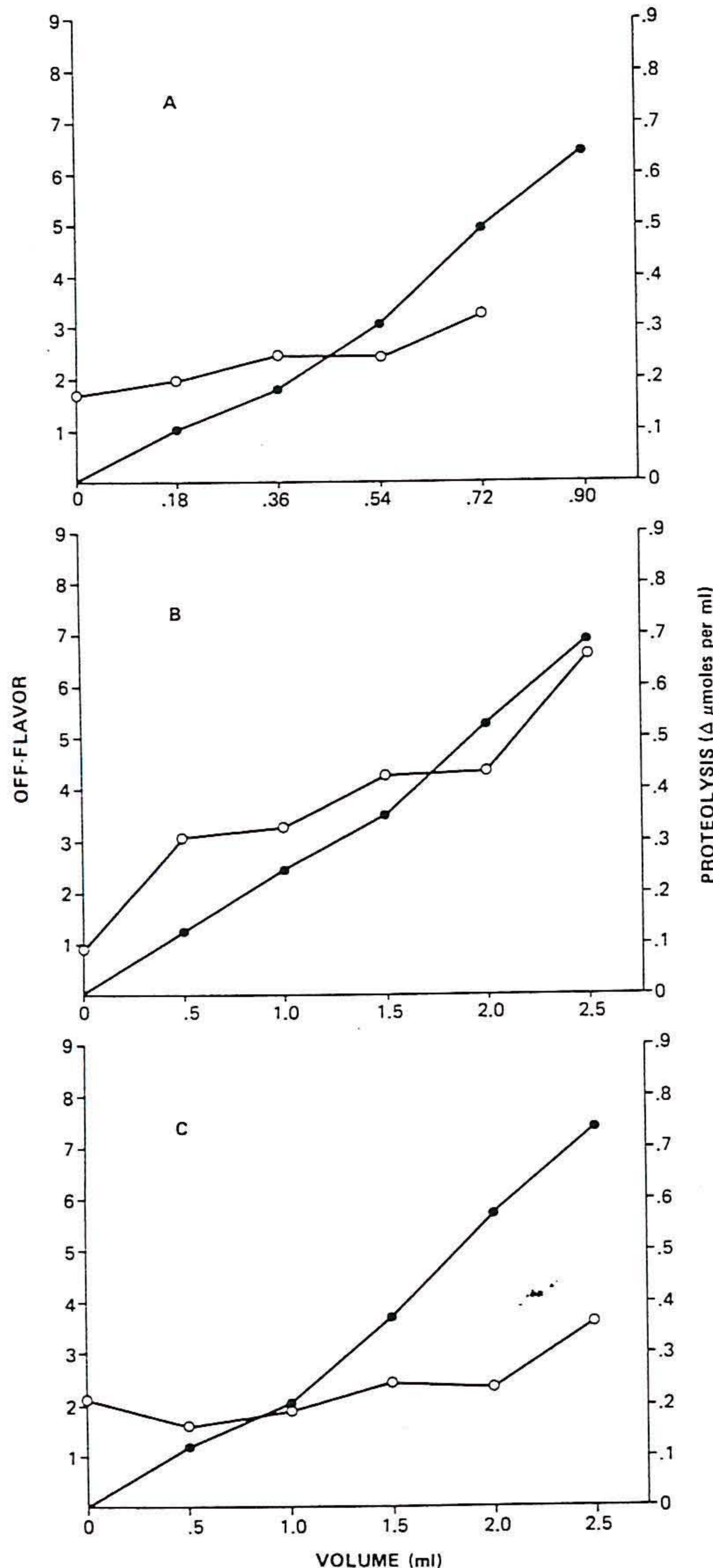

Figure 3. Effect of increasing volumes of cultural filtrate on proteolysis and intensity of off-flavor in pasteurized milk. a, strain 13; b, strain 15; c, strain 33. Off-flavor, o—o; proteolysis (Δ μmoles/ ml), •—•. Proteolysis was determined relative to the control at 35°C. For off-flavor and proteolysis, means were for three determinations. Standard errors of each mean for off-flavor and proteolysis were for enzyme 13, .304 and .015; for enzyme 15, .603 and .013; and for enzyme 33, .334 and .018.

TABLE 3. Proteolysis associated with significant off-flavor.[a]

Milk	Enzyme	Proteolysis (Δ μmoles/ml)
UHT	13	.554
UHT	15	.355
UHT	33	.289
Pasteurized	13	.499
Pasteurized	15	.355
Pasteurized	33	.746

[a]Significance at 5% was relative to controls lacking enzyme.

highest concentration of enzyme. Because of the increased resistance of pasteurized milk to proteolytic action, larger volumes of cultural filtrate as compared to UHT milk (.54, 1.0, and 2.5 ml for strain 13, 15, and 33, respectively) could be added to 150 ml of milk with no apparent change in flavor.

Coagulation was extensive when pasteurized milk was treated with the highest concentration of enzyme 13, and this sample, therefore, was not submitted for sensory evaluation of off-flavor. Coagulation was slight also with highest concentrations of enzymes 15 and 33. No thickening or coagulation was observed in UHT milk at any enzyme concentration.

The amount of proteolysis in UHT milk required for off-flavor to differ ($P<.05$) from coded controls was different for each of the enzymes (Table 3). For pasteurized milk, amounts for strains 13 and 15 were almost identical to those for UHT milk. In contrast, off-flavors were not detected in pasteurized milk with enzyme 33 until a greater than two-fold increase in proteolysis over that in UHT milk had been obtained (Table 3).

Figure 4 shows electrophoretic profiles of caseins isolated from UHT (a to g) and pasteurized (h to n) milk which had been treated with different amounts of cultural filtrate from strain 13. Little difference was observed between untreated UHT (a) and untreated pasteurized (h) milk with the exception that protein bands corresponding to κ-casein appeared to be less distinct in untreated UHT milk.

Increasing concentrations of enzyme had little effect on the electrophoretic profile of either UHT (Figure 4, c to g) or pasteurized (Figure 4, j to n) milk. Densiometric scans of the gels indicated that there was no measureable decrease in the relative amounts of α_s, β, or κ-casein (data not shown). For both types of milk, treatment with protease resulted in development of a slow moving band migrating close to the top of the gels (Figure 4, arrows). This band was detected in the controls but appeared to increase in intensity with increased protease concentration. This band, which could not be quantified accurately, was more clearly
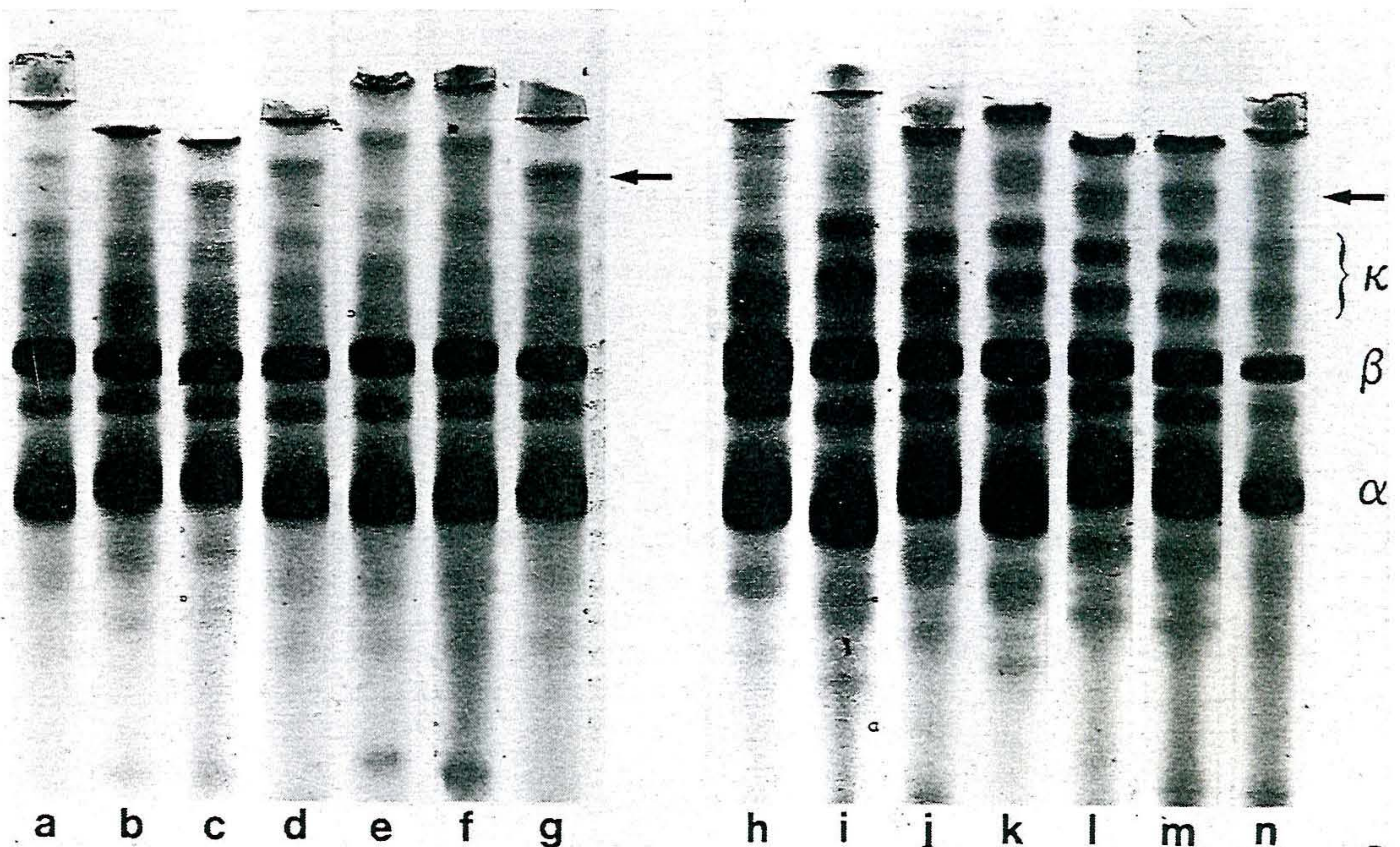

Figure 4. Electrophoretic profiles of caseins isolated from UHT and pasteurized milk treated with cultural filtrate from strain 13. a and b, UHT milk controls at 4 and 35°C; c to g, UHT milk treated with .07, .14, .21, .28, and .35 ml of cultural filtrate; h and i, pasteurized milk controls at 4 and 35°C; j to n, pasteurized milk treated with .18, .36, .54, .72, and .90 ml of cultural filtrate.

defined in UHT than in pasteurized milk.

Similar experiments with caseins isolated from UHT and pasteurized milk treated with cultural filtrate from strains 15 and 33 gave essentially the same results.

DISCUSSION

This study represents the first reported attempt to associate off-flavor development in milk with proteolysis measured as the release of TCA-soluble free amino groups. The most interesting finding was that with all three enzymes, proteolysis could be measured in both UHT and pasteurized milk before significant off-flavors were detected. Other tests which have been used to measure proteolysis in milk (7, 11, 13, 18) did not have this capability.

In addition, proteolysis necessary for significant off-flavor development in UHT and pasteurized milk was determined. This ranged from .289 to .554 and from .499 to .746 Δ μmoles per milliliter for UHT and pasteurized milk, respectively.

It might be possible to monitor stored UHT milk with TNBS. Small increases in TCA-soluble free amino groups during initial storage periods could be measured and this information used to predict how long the milk will keep before off-flavors might be expected to develop.

Proteolysis did not increase in a strictly linear fashion with increased enzyme concentration. Moreover, Adams et al. (1) demonstrated that susceptibility of UHT milk to action of proteolytic enzymes increased with age of the milk. Also, proteolysis required for off-flavor development appears to be different for each enzyme. Further studies with UHT milk stored over longer times are necessary to assess the role of TNBS as an indicator of shelf life.

Low levels of proteolysis detected by TNBS did not produce marked changes in the electrophoretic profile of UHT or skim milk. When off-flavors became significant, a faint, slow moving band in the controls had increased in intensity but could not be quantified accurately. Further, relative intensities of the casein bands were essentially unchanged even at the highest concentration of enzyme.

One of the problems that may be associated with use of TNBS to measure proteolysis in milk is the high content of TCA-soluble free amino groups in untreated milk. However, background values in raw, pasteurized, and UHT milk were similar. This was of particular interest since the UHT milk was obtained from a different source from pasteurized milk. Further studies are necessary to assess any natural variation in TCA-soluble free amino groups in raw and processed milk.

Increases in TCA-soluble free amino groups were significant when pasteurized milk was incubated at 4 and 35°C. These increases may result in part from action of native milk protease (6). Proteolysis due to psychrotrophic protease was expressed relative to controls at 35°C, and it is, therefore, not possible to assess the contribution of the native milk protease, if any, to development of off-flavor in pasteurized milk.

Pasteurized milk appears to be more resistant to proteolysis than UHT milk. The use of high temperatures during processing may lead to the exposure of new enzyme substrate sites on protein molecules. A similar exposure of new sites resulting from action of the proteases may explain the concave nature of the proteolysis curves in Figure 3.

Despite the greater resistance of pasteurized milk to proteolysis, off-flavors were detected in pasteurized milk treated with enzymes 13 and 15 at the same levels of proteolysis as UHT milk. This suggests that modifications in milk proteins brought about by UHT treatment have increased availability but not specificity of active sites. With enzyme 33, however, much higher proteolysis was required for off-flavor development in pasteurized than in UHT milk.

Gelation is a common problem in stored UHT milk (10). In this study, high enzyme concentrations resulted in clotting of pasteurized but not UHT milk. Samel et al. (15) reported that gelation only occurred in UHT milk if it was held at temperatures below 37°C. Possibly, incubation of UHT milk at 35°C in this study precluded gel formation.

In conclusion, the data reported here have indicated that since TNBS can be used to detect proteolysis in milk prior to development of off-flavors, it may be useful as an indicator of shelf life. Further studies involving long term storage of UHT milk are necessary to confirm this possible application.

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