A rapid means to detect the presence of protease activity in raw milk could be useful in predicting keeping ability of products made from that milk. A 30-min assay has been developed and compared with three other methods of detecting protease. Casein, [methyl-\(^{14}\)C]-methylated-\(\alpha\) was purchased from a radioisotope supplier. Concentrations of substrate from 2 to 20 nCi gave counts per minute, which increased linearly when counted with the Charm analyzer. There was not a significant difference in counting times of 10, 20, or 30 min. A mixture of sodium acetate and acetic acid precipitated nonhydrolyzed substrate with an efficiency of 97%. Comparison of the \(^{14}\)C casein assay, a casein fluorescein isothiocyanate assay, trinitrobenzenesulfonic acid procedure, and the Hull procedure using protease from psychrotrophic bacteria revealed that the \(^{14}\)C casein and casein fluorescein isothiocyanate methods were roughly equivalent and that the radiometric procedure was 10 times more sensitive than the trinitrobenzenesulfonic acid assay. The radiometric procedure was approximately \(10^4\) times more sensitive than the Hull procedure. The \(^{14}\)C casein and casein fluorescein isothiocyanate methods were similar in time required, about 30 min, while the trinitrobenzenesulfonic acid assay and Hull method required about 1 h plus reagent preparation time.

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The \(^{14}\)C casein procedure was most expensive per test; the other three were cheaper and similar to each other in cost.

INTRODUCTION

The adverse effects of proteases from psychrotrophic bacteria on milk and dairy products has been documented in many reviews (4, 5, 14). Proteolytic activity of the milk appears to be a more useful predictor of shelf-life than the quantity of psychrotrophic bacteria present (2, 9). Measurements of proteolysis have correlated well with sensory data (9, 10). Mitchell and Ewings (16) demonstrated correlation \((r = .86)\) between gelation time of UHT processed milk and level of protease activity. They could not, however, measure the minimum amount of protease that led to gelation within 5 mo of storage at 23°C but had to estimate threshold limits by dilution of more concentrated enzyme. They concluded that there is a need for an objective, precise, and sensitive method to measure protease activity in milk destined for UHT processing.

Drucker (7) reported on a sensitive radiochemical assay for proteolytic activities in 1972. Donnelly et al. (6) used a radiolabeled casein preparation to study plasmin activity. Both used radioisotope that had been prepared in the researchers’ laboratory. One limitation of the radioisotopic enzyme assays has been the lack of a commercially available labeled substrate. This has recently been overcome, and research in the author’s laboratory has evaluated such substrate for determination of bacterial protease in milk (17). Special precautions must be used in order to limit complications of high blank values.

The Charm procedure to quantify antibiotics in milk is widely used in dairy processing plants throughout the world (personal communications, Shirley Charm). The current research was undertaken to determine if the Charm analyzer...
could be used in a method to indicate proteolytic activity of milk. The method so developed was compared with three other methods of measuring proteolysis using three different psychrotrophic bacteria that had been isolated from raw milk and were capable of producing heat-resistant proteases.

### MATERIALS AND METHODS

#### Determination of Counting Efficiency

Casein, \([\text{methyl-}^{14}\text{C}]\)methylated-\(\alpha\) was obtained from New England Nuclear (Boston, MA). This radiolabeled preparation had a specific activity of approximately 2 \(\mu\text{Ci}/\text{mg}\) and was in .01 M sodium phosphate buffer at pH 7.2. The isotope was stored frozen until used. The final substrate was prepared by diluting 1 \(\mu\text{Ci}\) to a final volume of 5 ml with .01 phosphate buffer, pH 7.2. Quantities ranging from 0 to 100 \(\mu\text{l}\) of this substrate were pipetted into microcentrifuge tubes. Sterilized rehydrated NDM (RNDM) (10% wt/vol) was added to bring the final volume to 200 \(\mu\text{l}\). After mixing, the entire contents of the tube was transferred to a metal planchet (Penicillin Assays, Inc., Malden, MA) and dried in a thin film with very slight warming. Following drying, the planchet, containing dried substrate in milk, was counted in a Charm Test Analyzer, Model 160 (Penicillin Assays, Inc., Malden, MA) for 30 min in 10-min intervals. The counter was zeroed for background interference. Counts per minute were reported. Counting efficiency was determined based on specific activity information obtained from the supplier.

#### Precipitation Efficiency

The experiment described previously was repeated except that final volumes of substrate and RNDM were 100 \(\mu\text{l}\). One hundred microliters of a 50:50 mixture of 10% acetic acid and 1 N sodium acetate (12) was added to precipitate unhydrolyzed casein and the mixture centrifuged at 13,000 \(\times\) g for 5 min at room temperature in a microcentrifuge. One hundred microliters of the supernatant fluid was transferred to a metal planchet and the procedure repeated as previously described. Efficiency of precipitation was determined by counting 100 \(\mu\text{l}\) of a mixture of 50 \(\mu\text{l}\) substrate and 150 \(\mu\text{l}\) RNDM. Estimates of counts for other quantities of substrate were made by calculation.

#### Preliminary Enzyme Evaluation

Prior to the full experiment using several enzymes and different methods of analysis, purified enzyme from an unidentified psychrotrophic bacteria isolated from raw milk (8) was evaluated at levels of 0 to .05 units (1 unit is equivalent to the color of 1 \(\mu\text{mol}\) of tyrosine released/min at pH 7.5 and 30°C). Fifty microliters of substrate and 50 \(\mu\text{l}\) of enzyme prepared in RNDM were incubated at 30°C for 10 min. Following incubation, 100 \(\mu\text{l}\) of acetic acid-sodium acetate was added and the mixture was centrifuged as before. The rest of the procedure was as previously described.

#### Production of Enzyme

Three psychrotrophic bacteria \([\text{Pseudomonas fluorescens} 27 (P27); \text{Acinetobacter 32 (A32)}; \text{and an unidentified species (U7)}]\), which had previously been isolated from raw milk (8), were grown on the surface of dialysis membrane resting on the surface of peptone agar as described by Christen and Marshall (3). Enzyme was harvested and sterilized by membrane filtration as previously described (3). The preparation was partially purified by passing through a 90 x 2-cm Sephadex G-200 column using .1 M Tris buffer at pH 7.4. Ten-milliliter fractions were collected. Proteolytic activity was determined by agar diffusion in skim milk agar as described by Christen and Marshall (3). Proteolytic fractions were pooled and lyophilized. Enzyme activity of this preparation was determined based on the Hull test (11). Two milligrams of freeze-dried preparation were suspended in 2 ml of .02 M phosphate buffer at pH 7.5. One milliliter of enzyme was added to 9 ml of 3.5% sodium caseinate solution (containing 5 mM CaCl\(_2\)) and incubated at 30°C for 60 min. Following incubation, 10 ml of .7 N TCA were added to 5 ml of sample plus 1 ml of \(\text{H}_2\text{O}\) and allowed to stand for 10 min prior to filtration through No. 40 Whatman filter paper. The rest of the procedure was as described by Hull. Blanks were made by adding TCA immediately after enzyme addition. One enzyme activity unit was defined as the amount of enzyme that would hydrolyze casein to produce the color equivalent of 1 \(\mu\text{mol}\) of tyrosine/min at pH 7.5 and 30°C. Activity of each enzyme preparation used for subsequent evaluation was based on its activity determined by the Hull test.
Comparison of Protease Methods

Serial dilutions of enzyme were prepared to obtain enzyme activity based on the Hull procedure of approximately 0.2 units/ml of total assay mixture in the most concentrated sample and 0.025 units/ml in the least concentrated. The same concentrations were used within bacterial protease type but there was variation between types. Controls, containing RNDM in place of enzyme, were used in each assay. All analyses were replicated. The enzyme assay using radiolabeled casein was performed as described under the preliminary enzyme analysis section. Incubation was at 30°C for 10 min and counting time was 10 min.

Trinitrobenzenesulfonic acid (TNBS) and Hull analysis were performed using RNDM as substrate. One milliliter of enzyme was added to 9 ml of RNDM and incubated at 30°C for 30 or 60 min, respectively. Nonhydrolyzed protein was removed by TCA precipitation and the increase in free amino groups determined by either the TNBS procedure (15) or the Hull procedure (11). Glycine was used as the standard in the TNBS procedure while tyrosine was used in the Hull.

A casein fluorescein isothiocyanate (FITC) procedure was also used (17). Briefly, this procedure used 0.25% (wt/vol) FITC (Type III; Sigma, St. Louis, MO) in 0.01 M phosphate buffer (pH 7.2). Forty microliters of FITC substrate were mixed with 10 μl of enzyme in RNDM and incubated for 10 min at 30°C. Following incubation 80 μl of 5% TCA was added and nonhydrolyzed substrate was removed by centrifugation (13,000 × g) for 5 min. Eighty microliters of supernatant portion was diluted to 2 ml with 0.3 M phosphate buffer (pH 8.5). Fluorescence was measured at 525 nm with excitation at 367 nm.

Statistical Analysis

Where appropriate, data were analyzed using the general linear models procedure of SAS (Cary, NC). Significance was established at α = 0.05%. The data from each protease assay procedure were linearized using a y^B transformation and then analyzed according to the procedure of Deming (13) using a program written in Basic for a Radio Shack TRS80 Model III microcomputer. Detectability of different methods was calculated by s_e/slope; both values were obtained from the Deming analysis (13). Classical regression analysis and correlation analysis were also performed using SAS.

RESULTS AND DISCUSSION

Counting Efficiency

Variation in substrate concentration from 0 to 20 nCi accounted for 98% of the total variation in counts per minute when the variables of substrate concentration (eight levels), counting time (three levels), replication (three times), and the interaction of substrate concentration and counting time were included in analysis of variance. Whether the samples were counted 10, 20, or 30 min prior to calculation of counts per minute had no effect on the results (the F value rounded to the nearest hundredth was 0). Each substrate gave mean counts per minute, which were significantly different from all others (Table 1).

When the same data were analyzed with counting efficiency as the dependent variable, time of counting again had no effect on results. Quantity of substrate had a statistically significant effect, although the practical significance of a difference in counting efficiency between 6 to 8% is questionable (Table 1). All values for counting efficiency fall within the range expected for a counter of this type.

Precipitation Efficiency

Initially an attempt was made to remove the nonhydrolyzed labeled casein by TCA precipitation as is commonly done in protease assays. The TCA, however, reacted with the metal planchets giving unacceptable results. Because only the large casein molecule needed to be removed from the hydrolysis products, the reagent used in the Aschaffenburg and Drewry (1) procedure for determination of noncasein nitrogen was tried. A summary of the results of this process is given in Table 2. Precipitation efficiency ranged from 92.7 to 98.5%, with that for 10 nCi of substrate being greater than 97%. This was much improved over the results obtained previously with TCA and liquid scintillation counting (17). Although the average precipitation efficiency was higher with small quantities of substrate, greater quantities were precipitated with more precision than were the lesser quantities. This better precision is
TABLE 1. Mean counts per minute (cpm) and counting efficiency for [14C] casein substrate.

<table>
<thead>
<tr>
<th>Quantity of substrate (nCi)</th>
<th>cpm</th>
<th>SD</th>
<th>CV</th>
<th>Expected dpm</th>
<th>Counting efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>288</td>
<td>14.6</td>
<td>15.2</td>
<td>4400</td>
<td>6.60ab</td>
</tr>
<tr>
<td>4</td>
<td>522</td>
<td>5.8</td>
<td>3.3</td>
<td>8800</td>
<td>6.00a</td>
</tr>
<tr>
<td>6</td>
<td>787</td>
<td>6.2</td>
<td>11.1</td>
<td>13,200</td>
<td>6.00a</td>
</tr>
<tr>
<td>8</td>
<td>1291</td>
<td>66.9</td>
<td>15.5</td>
<td>17,600</td>
<td>4.30c</td>
</tr>
<tr>
<td>10</td>
<td>1561</td>
<td>57.8</td>
<td>11.5</td>
<td>22,000</td>
<td>7.10bc</td>
</tr>
<tr>
<td>15</td>
<td>2161</td>
<td>43.0</td>
<td>8.0</td>
<td>33,000</td>
<td>6.50ab</td>
</tr>
<tr>
<td>20</td>
<td>3513</td>
<td>94.1</td>
<td>8.0</td>
<td>44,000</td>
<td>8.00d</td>
</tr>
</tbody>
</table>

Means in the same column followed by the same superscript are not significantly different (P<.05).

1 n=9.

2 Disintegrations per minute (1 nCi = 2200 dpm).

3 Counting efficiency: (cpm/dpm) × 100.

apparent from the decline in magnitude of the coefficient of variation (%) as substrate increased from 2 to 20 nCi.

When the variance in counts per minute results were distributed among the variables of substrate concentration (eight levels), time of counting (three levels), replication (three times), and interaction between substrate concentration and counting time, substrate concentration accounted for more than 99% of the variation. Each substrate level was significantly different from all others (Table 2). Counting time had essentially no effect on the overall variance (F = .02).

When counts per minute after removal of nonhydrolyzed substrate were plotted against nanocuries of substrate added initially, the plot departed from linearity near 15 nCi of substrate (Figure 1). Because of this and because of the cost of the substrate, 10 nCi of substrate

TABLE 2. Mean counts per minute (cpm) and precipitation efficiency when a mixture of acetic acid and sodium acetate was used to precipitate nonhydrolyzed casein.

<table>
<thead>
<tr>
<th>Quantity of substrate (nCi)</th>
<th>cpm</th>
<th>SD</th>
<th>CV</th>
<th>Precipitation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.29</td>
<td>.23</td>
<td>10.0</td>
<td>98.5</td>
</tr>
<tr>
<td>4</td>
<td>7.81</td>
<td>2.11</td>
<td>27.0</td>
<td>97.3</td>
</tr>
<tr>
<td>6</td>
<td>11.39</td>
<td>2.24</td>
<td>19.7</td>
<td>97.8</td>
</tr>
<tr>
<td>8</td>
<td>15.64</td>
<td>2.56</td>
<td>16.4</td>
<td>97.7</td>
</tr>
<tr>
<td>10</td>
<td>23.12</td>
<td>.70</td>
<td>3.0</td>
<td>97.1</td>
</tr>
<tr>
<td>15</td>
<td>39.97</td>
<td>1.35</td>
<td>3.4</td>
<td>96.7</td>
</tr>
<tr>
<td>20</td>
<td>115.98</td>
<td>3.07</td>
<td>2.6</td>
<td>92.7</td>
</tr>
</tbody>
</table>

Means in the same column with the same superscripts are not significantly different (P<.05).

1 n = 9.
MEASURING PROTEASE ACTIVITY

was used in all subsequent experiments. This substrate level had given good counting and precipitation efficiency.

Preliminary Enzyme Assay

The Hull test on the lyophilized enzyme preparation revealed that each milligram of powder contained about $80 \times 10^{-4}$ units of activity. Serial dilutions (1:2 each time) were made to produce enzyme concentrations from 5 to $80 \times 10^{-4}$ units/ml. An enzyme solution containing $80 \times 10^{-4}$ units/ml (10 mg enzyme) produced absorbance by the Hull test after 60 min of incubation that was twice the background (.154 vs .068). Fifty microliters of each dilution was analyzed by the $[^{14}C]$casein procedure with a 10-min incubation and 10-min counting time. Table 3 shows the mean results of three replications of this experiment. There was not a significant difference between replications. Additionally, each level of enzyme produced results significantly different from all others. Fifty microliters of the sample containing $5 \times 10^{-4}$ units/ml is 320 times less enzyme protein than is 1 ml of a sample containing $80 \times 10^{-4}$ units. The results of the $[^{14}C]$casein assay were obtained within 30 min. The $[^{14}C]$casein assay using the Charm analyzer appears to have the desired sensitivity, repeatability, and speed that was being sought. Therefore, further investigation of the procedure was initiated.

Comparison of Protease Methods

Table 4 contains the statistics derived from the Deming analysis of the $[^{14}C]$casein method compared with FITC, TNBS, and Hull procedures. In all cases, the $[^{14}C]$casein method was able to detect lower amounts of enzyme activity as demonstrated by the smaller detectability values for that method. Figures 2 through 4 indicate the relationship between the $[^{14}C]$casein procedure and the other procedures. The data points are those derived by the test procedure and have not been linearized. In every instance, the methods of detecting protease activity were highly correlated.

Figures 5 through 8 show the relationship between the amount of enzyme activity and the results of each protease assay. The regression equation that corresponds to each figure is given in the legend for that figure. The different symbols relate to the three different protease

---

TABLE 3. Initial investigation of $[^{14}C]$casein assay using protease from a psychrotrophic bacteria.

<table>
<thead>
<tr>
<th>Units (× 10^{-4})</th>
<th>Mean cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>157.6a</td>
</tr>
<tr>
<td>40</td>
<td>123.6b</td>
</tr>
<tr>
<td>20</td>
<td>93.8c</td>
</tr>
<tr>
<td>10</td>
<td>67.8d</td>
</tr>
<tr>
<td>5</td>
<td>46.5e</td>
</tr>
<tr>
<td>0</td>
<td>28.8f</td>
</tr>
</tbody>
</table>

a,b,c,d,e,f Means followed by different superscripts are significantly different ($P<.05$).

1 Unit = Color equivalent of 1 μmol tyrosine released/min at 37°C and pH 7.

2 n = 3.

3 Counts per minute.

<table>
<thead>
<tr>
<th>Methods compared</th>
<th>n</th>
<th>B Value</th>
<th>Error</th>
<th>Slope</th>
<th>Detectability $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C Casein</td>
<td>36</td>
<td>1.2</td>
<td>35.2</td>
<td>3.8</td>
<td>9.4</td>
</tr>
<tr>
<td>FITC</td>
<td>.8</td>
<td>4</td>
<td>.3</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td>$^{14}$C Casein</td>
<td>36</td>
<td>1.6</td>
<td>134.9</td>
<td>440.5</td>
<td>.31</td>
</tr>
<tr>
<td>TNBS</td>
<td>1.8</td>
<td>1.3</td>
<td>.36</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>$^{14}$C Casein</td>
<td>18</td>
<td>2.2</td>
<td>443.9</td>
<td>$2 \times 10^4$</td>
<td>.02</td>
</tr>
<tr>
<td>Hull</td>
<td>.1</td>
<td>.7</td>
<td>.001</td>
<td>700</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Detectability = error/slope. General equation: $y^B = ax + b$.

samples. There is a very good linear relationship between the amount of enzyme activity added and the results of the TNBS and Hull method. The high $R^2$ obtained with the Hull procedure was expected, because the results of that procedure were used to calculate the enzyme activity units. Nearly 80% of the variation in the results of the $^{14}$C casein procedure was explained by the increase in enzyme activity. Regression of the results obtained with the FITC procedure against increase in enzyme activity explained less of the variation. However, most of this variation was associated with the protease of Unknown 7. The sample size for the $^{14}$C casein procedure was 20 times less than that used in the TNBS and Hull analysis; incubation time was 10 min vs. 30 min for TNBS and 60 min for Hull. The FITC procedure used a sample size five times smaller than the $^{14}$C casein procedure, but incubation times were the same. The reagent cost for the $^{14}$C casein procedure, the FITC procedure, the TNBS procedure, and the Hull procedure decrease in the order given. $^{14}$C Casein costs
Figure 4. Comparison of protease activity measured by the $^{14}$C casein procedure and the Hull procedure. Actual data have been plotted ($n = 18; r = .847; P = .001$).

Figure 5. Protease activity measured by the $^{14}$C casein procedure plotted against calculated units of protease activity. Linear regression statistics: y-intercept = 25.9, slope = 2.7, $R^2 = .78$, standard deviation = 16.78 ($\oplus$ = protease from P27; $\triangle$ = protease from A32; $\varnothing$ = protease from U7).

Figure 6. Protease activity measured by the FITC procedure plotted against calculated units of protease activity. Linear regression statistics: y-intercept = 21.2, slope = 1.7, $R^2 = .43$, standard deviation = 22.00 ($\oplus$ = protease from P27; $\triangle$ = protease from A32; $\varnothing$ = protease from U7).

Figure 7. Protease activity (micromoles of free amino groups per milliliter) measured by the TNBS procedure plotted against calculated units of protease activity. Linear regression statistics: y-intercept = 1.7, slope = .15, $R^2 = .86$, standard deviation = .72 ($\oplus$ = protease from P27; $\triangle$ = protease from A32; $\varnothing$ = protease from U7).
Figure 8. Protease activity (micromoles of tyrosine per milliliter) measured by the Hull procedure plotted against calculated units of protease activity. Linear regression statistics: y-intercept = 1.2, slope = .06, $R^2 = .97$, standard deviation = .12 ($\Delta$ = protease from P27; $\odot$ = protease from A32; $\odot$ = protease from U7).

about $3.00 per test. Cost of the other substrates is less than $.10.

Use of the Charm test analyzer to determine protease activity in milk appears to have merit and deserves further investigation. It provides a rapid, although not inexpensive method, to determine protease activity using equipment currently available in many dairy processing plants. Further investigation will be necessary to determine if knowledge of the amount of protease present can be related to keeping ability of the products made from the milk or to the bacterial quality of the milk prior to processing.

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