COMPARISON OF PROTEINASE ASSAY METHODS AND IDENTIFICATION OF PSEUDOMONADS IN MILK

BY

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ABSTRACT

This thesis is divided into two chapters summarized separately below.

(1) Four methods (absorbance at 280 nm; the Lowry method; the fluorescamine method and the trinitrobenzenesulfonic acid method) for determining hydrolysis of milk proteins were compared. Each method was applied to the trichloracetic acid soluble fraction of milk protein, which had been digested with trypsin for various periods of time. Detectability was measured as the ratio between standard error of estimate and slope calculated from the linear regression analysis of Deming for cases when both variables were subject to error. Although it was nondimensional, the detectability thus calculated was simple and reliable for comparing assay methods which were based on different analytical principles. Detectability as well as the detection limit measured according to Schwerdtfeger showed that, of the methods compared, the fluorescamine method was most reliable and sensitive.

(2) Proteolytic pseudomonads isolated from raw milk were classified by numerical taxonomy. Unweighted pair-group average-linkage cluster analysis was used to cluster 49 bacterial strains, of which 26 were *Pseudomonas* species as described in the Bergey's Manual of Determinative Bacteriology, based on 52 characters. The milk isolates resided in two clusters: one containing *P. fluorescens* and the other *P. fragi*.

The isolates identified with P. fluorescens could hydrolyze milk proteins, butterfat; could produce phospholipase. The P. fragi-like isolates could hydrolyze milk proteins, butterfat; but could not produce phospholipase and fluorescent pigment. Studies, on hydrolytic characters of milk isolates, showed that the nature of the substrate and conditions under which the test was being conducted were critical. No relationship was found between proteolytic psychrotrophs populations and proteolysis in milk stored at 4 C. Electropherograms showed that β-casein was more susceptible to degradation by milk pseudomonads.

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CHAPTER 1

Comparison of four methods for determining proteolytic activity in milk

INTRODUCTION

As a consequence of changing socio-economic conditions and production methods, there is a trend toward a longer period of time elapsing between milking and processing of milk. These extended periods of time permit the growth of psychrotrophic bacteria in raw milk (Cousin, 1982). Proteinases produced by many strains of psychrotrophic Pseudomonas species are extremely heat resistant (Adams et al., 1976) and have been shown to decrease the shelf life of dairy products (Cousin, 1982).

The Hull method (Hull, 1947) has been used to measure proteolytic activity in milk in most studies (Adams et al., 1975; Gebre-Egziabher et al., 1980; Richter et al., 1979). This colorimetric method measures the acid-soluble tyrosine and tryptophan released during proteolysis. Cogan (1977) indicated that the measurement of proteolysis by means of the increase in trichloracetic acid (TCA) soluble tyrosine and tryptophan has some disadvantages since little TCA-soluble tyrosine and tryptophan may be released during proteolysis. The need for a rapid, sensitive and reliable method for determing proteolysis has been stressed (Law, 1979; Richter et al., 1979).

Juffs (1973) evaluated the Lowry modification of the Folin procedure (Lowry et al., 1951), expressing proteolysis in terms of tyrosine concentration. Alichanidis and Andrews (1977) studied the proteolytic activity of the extracellular proteinase produced by a Pseudomonas strain using a modification of the Lowry method. Richardson and Te Whaiti (1978) used absorbance

at 280 nm (A_{280}) to assay proteinase activity. Trinitrobenzenesulfonic acid (TNBS), a nonspecific amino acid reagent, was used to measure proteolysis in milk (McKellar, 1981). Snoeren et al. (1979) and Snoeren and Both (1981) determined the nitrogen content in different fractions as ammonia by a rapid colorimetric procedure (Koops et al., 1975). Griffiths et al. (1981) used a proteinase assay based on the ability of a variety of proteinases to release a blue dye covalently attached to denatured collagen. Starch gel and polyacrylamide gel electrophoresis have been used to estimate degradation of casein components in raw milk (Juffs, 1975; Law et al., 1979). Chism et al. (1979) developed a sensitive assay for proteinases based on the reaction of amino groups of TCA-soluble peptides and amino acids with the fluorescamine. A micromethod, using fluorescamine, for the assay of proteolysis was developed by Castell et al. (1979). Fluorescamine reacts with free amino groups of amino acids and peptides liberated during proteolysis.

Although there have been many papers published for assaying proteolysis, there are only few papers in which objective and statistically reliable comparison of different methods are described. The papers to compare different methods reported by Schwabe (1973) and by Kas and Rauch (1982) are all without reliable supporting data. Schwerdtfeger (1977) compared the TNBS, Folin, turbidity, radial diffusion on gel and the anilinonaphthalene sulfonate fluorescence methods using a detection limit determined as the minimum amount of the

proteinases detectable. However, this method for comparison is subject of the analytical conditions especially incubation conditions. This method is also tedious since the amount of proteinases has to be changed until F-value becomes significant ($p < 0.01\%$; $n = 4$).

The object of this study was to evaluate four methods for measuring protein hydrolysis in milk in terms of their reliability and detectability. The methods compared were: absorbance at 280 nm (Juffs, 1973); the Lowry method (Lowry et al., 1951); a modification of the microfluorometric method of Castell et al. (1979); and the TNBS method (McKellar, 1981). These four methods have not been compared before in a statistically reliable way. Detectability calculated from the linear regression analysis of Deming (Wakkers et al., 1975) and the detection limit defined by Schwerdtfeger (1977) were used for reliable comparison.

LITERATURE REVIEW

A. Application of the fluorescamine method for assaying proteolysis in milk

Fluorescamine (Fluram) is a useful reagent for the detection of amino acids, peptides, proteins and other primary amines. Its reaction with primary amines is spontaneous at room temperature. The reaction products are fluorescent, while fluorescamine and its degradation products are non-fluorescent (Udenfriend et al., 1972). The simplicity and sensitivity of the fluorescamine method make it attractive for proteinase activity determination.

Schwabe (1973) described a fluorescent assay for determining cathepsin activity using fluorescamine. Chism et al. (1979) modified the method of Schwabe and applied it to assay proteinases in milk. This method involved the reactions of TCA-soluble peptides and amino acids with fluorescamine.

Pearce (1979) developed a method using fluorescamine to study the rennet action on milk. The change in fluorescence intensity brought about by rennet action was proportional to the amount of caseino-macropeptide released. Beeby (1980) also used fluorescamine to follow the action of chymosin on κ-casein.

B. Comparison of methods for determination of protein hydrolysis

When two or more methods for determination of protein hydrolysis exist, there arises at once the problem of comparing their relative merits.

According to Schwabe's (1973) finding, the fluorescamine method was one hundred times more sensitive than the Lowry method. Data from which the conclusion was drawn were not published.

Nakai et al. (1974) described a study on the application of fluorescamine in the analyses of peptides in column chromatographic fractions. The fluorescent assay was found to be approximately five times as sensitive as the ninhydrin method. However there was no data to support this conclusion.

Spencer and Spencer (1974) did a comparison of the ninhydrin assay method and the 1-anilino-8-naphthalenesulfonate (ANS) assay method. Proteolytic activity of an extract from pumpkin seeds using pumpkin seed globulin as a substrate was determined by the two methods. Fluorimetric activity was given as change in relative fluorescence unit. Activity by the ninhydrin method was given as change in absorbance at 570 nm. The ANS-fluorescence method was found to be approximately twenty times more sentive in terms of the change in fluorescence relative to the change in absorption per milligram extract protein.

Schwerdtfeger (1977) compared different methods for the assay proteinase activities by determining the minimum amount of the proteinases detectable by each method. The TNBS method was shown to be the most sensitive of all the methods studied.

Kas and Rauch (1982) detailed a radiometric method for the determination of proteolytic activities in food. Results obtained were compared with that determined by the Lowry method. Enzymatic activities were expressed in absorbance and counts per minutes. Through direct comparison of enzymatic activities measured by the two methods, the radiometric method was reported to be one hundred times more sensitive.

MATERIALS AND METHODS

Fluorescamine (Fluram) was obtained from Chemical Dynamics Corp. (South Plainfield, NJ). Trinitrobenzenesulfonic acid was obtained from Eastman Kodak (Rochester, NY) and Folin-Ciocalteau phenol reagent from Fisher Scientific (Fair Lawn, NJ). Trypsin (Type XI, diphenyl carbonyl chloride treated, from bovine pancreas) was obtained from Sigma Chemical Company (St. Louis, MO). All chemicals and reagents were of reagent grade.

A. Comparison of the proteinase assay methods

To assay the effect of trypsin on milk proteins, 2.5 ml of 0.02 M phosphate buffer (pH 6.6) containing trypsin (24.1 µg/ml) were added to 150 ml of 10% (w/v) reconstituted skim milk (RSM) in 0.2 M phosphate buffer (pH 6.6). The reaction was run at 40 C. At various time intervals (1-7 min), 5.0 ml of the reaction mixture were withdrawn and the reaction stopped by the addition of an equal volume of 24% (w/v) TCA. The mixture was centrifuged (10,000 x g, 5 min) and the supernatant filtered (Whatman No. 4). The filtrate was assayed with the following methods. For some samples it was necessary to dilute with 12% (w/v) TCA to yield on-scale readings.

1) Absorbance at 280 nm

Absorbance of the filtrates, which were diluted when necessary (usually diluted five times), was determined with a

Unicam SP-800B Spectrophotometer (Pye Unicam Ltd., Cambridge, England).

2) Lowry method

Filtrate (1.0 ml) was mixed with 5.0 ml of the alkaline copper tartrate reagent as described by Lowry et al. (1951). After 10 min, 0.5 ml of Folin-Ciocalteau reagent, 1.0 N in acidity, was added. After incubation at room temperature for 30 min, absorbance was measured at 700 nm with a Unicam SP-800B Spectrophotometer.

3) Trinitrobenzenesulfonic acid method

Filtrate (0.2 ml) was mixed with 2.0 ml of 0.2 M sodium borate buffer (pH 9.2) and 1.0 ml of 4.0 mM TNBS. After incubation at room temperature for 30 min, 1.0 ml of 2.0 M monobasic sodium phosphate containing 18 mM sodium sulfite was added. Absorbance, at 420 nm, was measured with a Unicam SP-800B Spectrophotometer.

4) Fluorescamine method

To 0.1 ml of filtrate, 0.3 ml of 3 M potassium phosphate dibasic was added followed by 0.15 ml of 0.03% (w/v) fluorescamine in acetone. The mixture was immediately mixed. To adjust the volume to cuvette size, 3.0 ml of distilled water were added. Fluorescence intensity (excitation wavelength at 395 nm; emmission wavelength at 480 nm) was measured with an Aminco Bowman 4-8202 spectrofluorometer (American Instrument

Co., Inc., Silver Spring, MD).

B. Statistical comparison of the proteinase assay methods

Data, in terms of absorbance (from A_{280}, Lowry and TNBS methods) and fluorescence intensity (from the fluorescamine method), were pooled. Deming's method (Wakkers et al., 1975), using a program written for a Monroe 1880-88 programmable calculator, was employed. Data obtained at various time intervals with one proteolysis assay method (eg. fluorometric) were compared with the data for the same time intervals from another proteolysis assay method (eg. TNBS). This analysis produced six regression lines (each regression compared two methods). With this program, the standard error of estimate and the slope of the regression line were calculated. The detectability of each proteolysis assay method was expressed as the standard error of estimate of a particular assay method divided by its slope ratio.

For each pair of methods for measuring different hydrolysis products (i.e. amino groups and aromatic groups), the data transformation of Fujii and Nakai (1980) was applied for linearization using a transformation equation of $y^B = ax$. Then, Deming's method was applied for comparison of the of the methods using y^B vs. x as variables.

C. Determination of the detection limit of the TNBS and

fluorescamine methods

The method according to Schwerdtfeger (1977) was followed. To 10 ml of 0.2 M borate buffer (pH 7.4) containing 0.05% $CaCl_2$ and 1.0 ml of 10% RSM, 10 ml of trypsin at various concentrations were added. The mixture was incubated at 35 C. After 24 h, the reaction was terminated by the addition of TCA (3.5% final concentration). The mixture was centrifuged (10,000 x g; 5 min) and the supernatent filtered (Whatman #4). The filtrate was assayed with the fluorescamine method and the TNBS method as described by Schwerdtfeger (1974). The detection limit was defined as the trypsin concentration required to yield significant difference ($P < 0.01\%$; $n = 4$) between samples before and after incubation (Schwerdtfeger, 1977).

RESULTS AND DISCUSSION

The results of comparison of the methods for determining proteinase activity are shown in Figures 1-6. Relationships between the fluorescamine method vs. the TNBS method (Fig. 1), and the Lowry method vs. the A_{280} method (Fig. 6) appear to be linear. This is reasonable since the first pair of methods are both determining amino groups while the second pair of methods are both for aromatic groups.

On the contrary, the relationship between the fluorescamine method and the Lowry method (Fig. 2) does not appear to be linear. Similar nonlinear relationships were observed between the fluorescamine method and the A_{280} method (Fig. 3), the TNBS method and the Lowry method (Fig. 4), and the TNBS method and the A_{280} method (Fig. 5). This curvilinearity may be a consequence of different hydrolysis products being detected by each method. For these pairs of methods the plots were linearized first, then the methods were compared by Deming's method. Linearization considerably improved the detectability of the Lowry method but not for the other methods (Table 1) probably because of considerable deviation of the Lowry method from linearity.

Table 1 shows that the fluorescamine method was more reliable than the other three methods (smaller standard deviation of the random errors in the method). The TNBS method was more reliable than the Lowry method or the A_{280} method as a measure of the degree of hydrolysis of milk proteins. The

Fig. 1. Comparison of the fluorescamine method with the TNBS method as a measure of protein hydrolysis by Deming's method. Absorbance and fluorescence intensity are expressed as the apparent absorbance and fluorescence intensity of the undiluted solution.

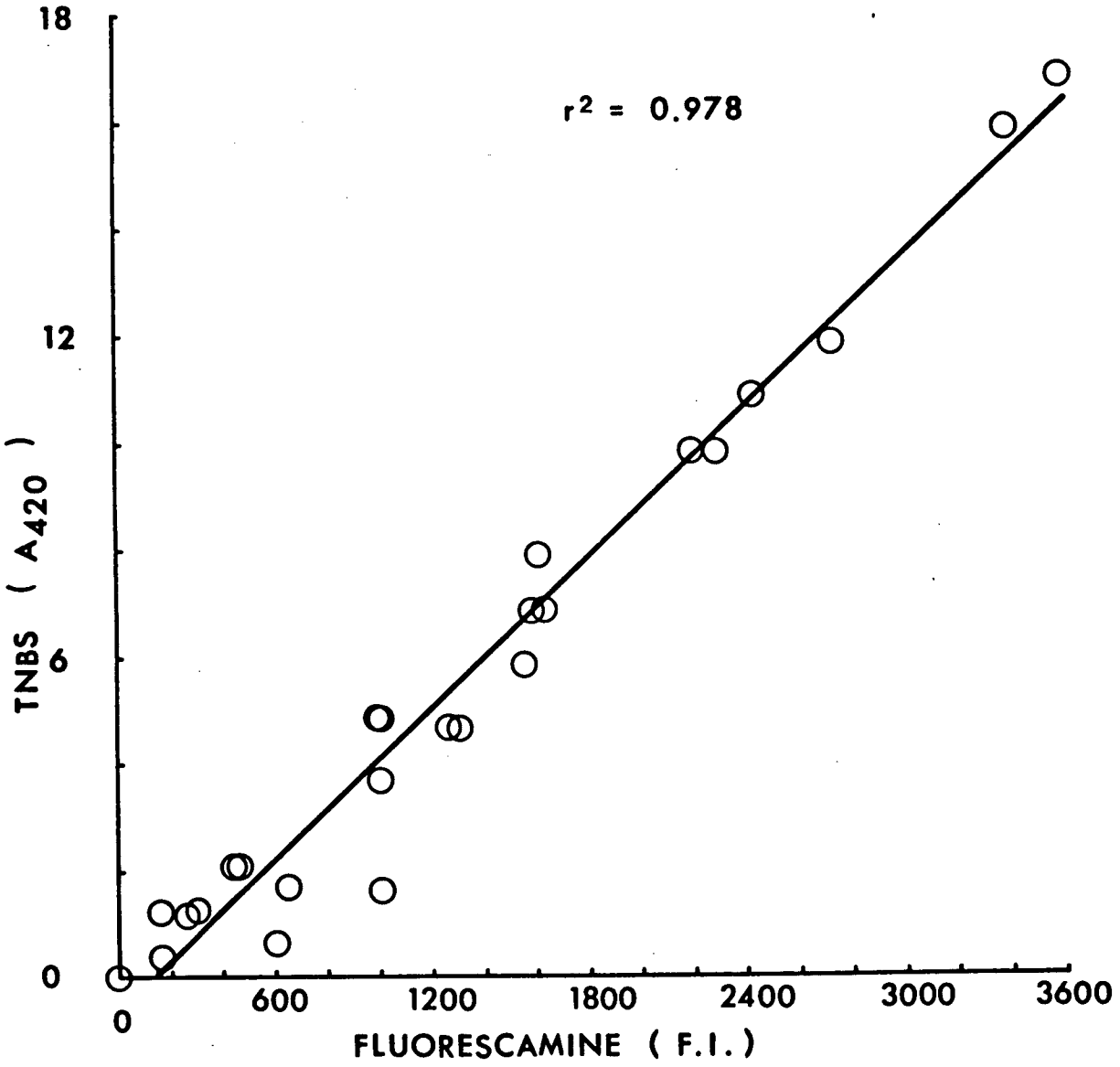

18
12
6
0
TNBS (A_{420})
r^2 = 0.978
0
600
1200
1800
2400
3000
3600
FLUORESCAMINE (F.I.)

Fig. 2. Comparison of the fluorescamine method with the Lowry method as a measure of protein hydrolysis by Deming's method. Absorbance and fluorescence intensity are expressed as the apparent absorbance and fluorescence intensity of the undiluted solution. The broken line represents the transformed data from the Lowry method vs. the fluorescamine method.

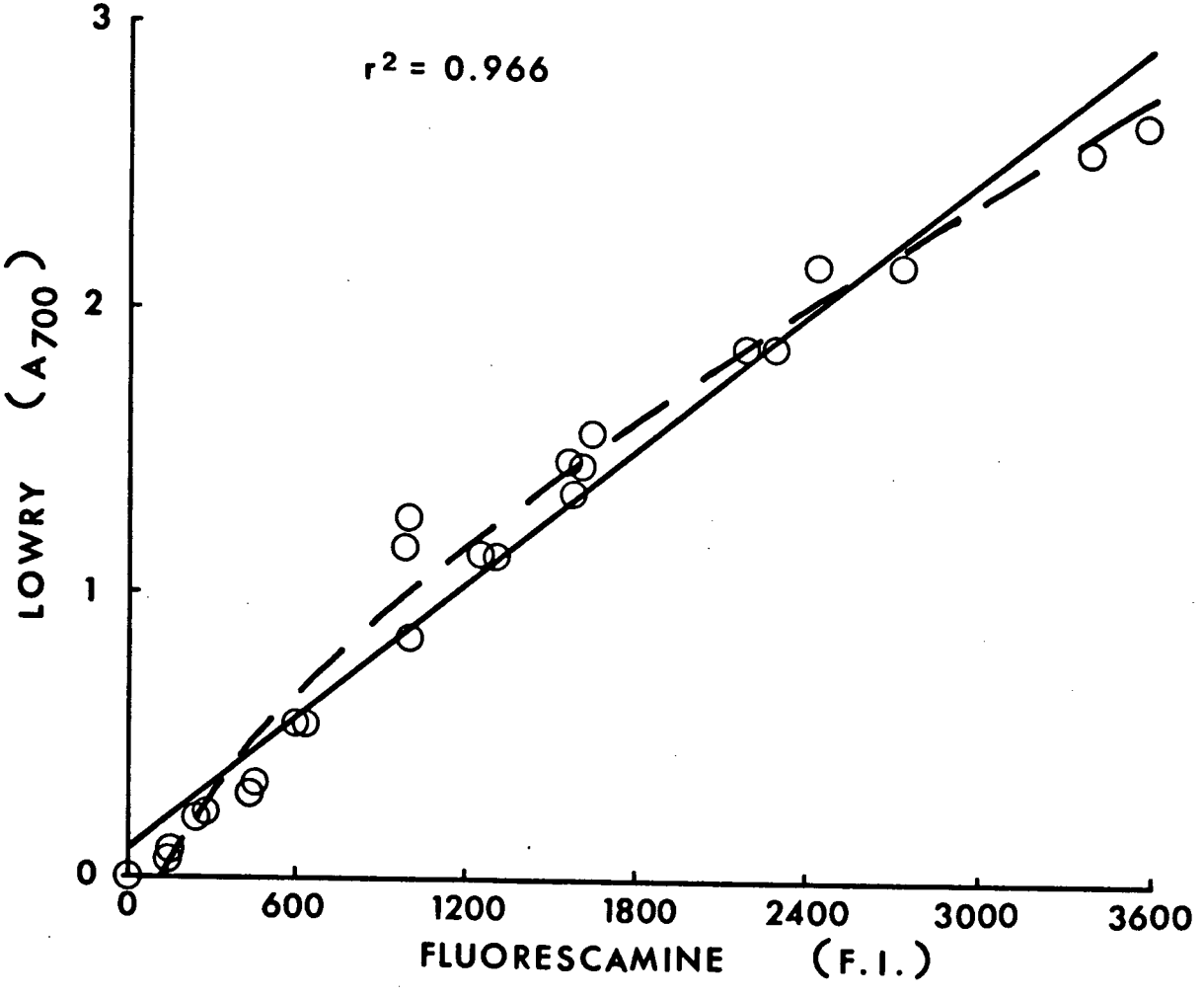

r² = 0.966
LOWRY (A700)
FLUORESCAMINE (F.I.)
0
1
2
3
0
600
1200
1800
2400
3000
3600

Fig. 3. Comparison of the fluorescamine method with the A_{280} method as a measure of protein hydrolysis by Deming's method. Absorbance and fluorescence intensity are expressed as the apparent absorbance and fluorescence intensity of the undiluted solution. The broken line represents the transformed data from the A_{280} method vs. the fluorescamine method.

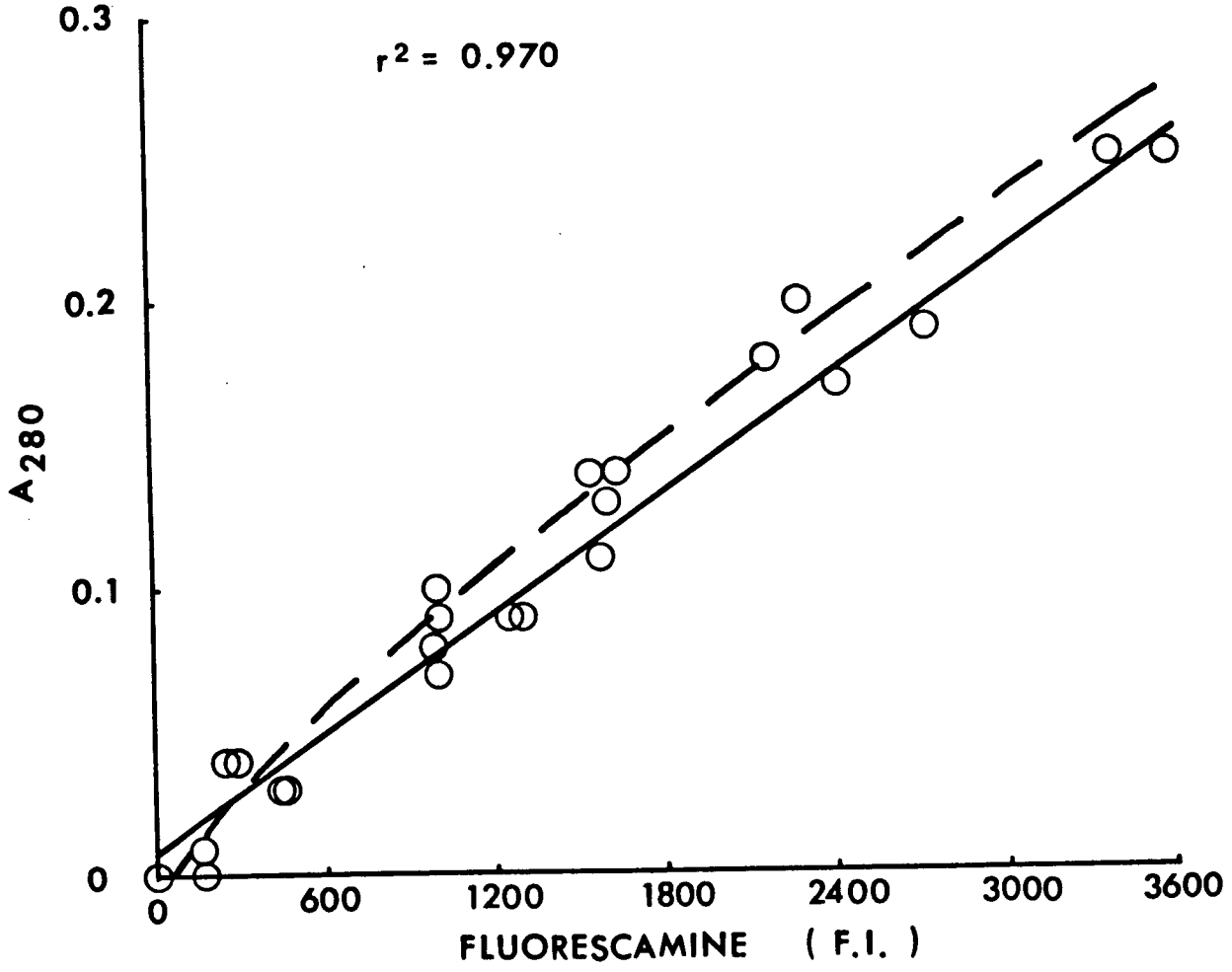

0.3
0.2
0.1
0
A_{280}
$r^2 = 0.970$
0
600
1200
1800
2400
3000
3600
FLUORESCAMINE (F.I.)

Fig. 4. Comparison of the TNBS method with the Lowry method as a measure of protein hydrolysis by Deming's method. Absorbance is expressed as the apparent absorbance of the undiluted solution. The broken line represents the transformed data from the Lowry method vs. the TNBS method.

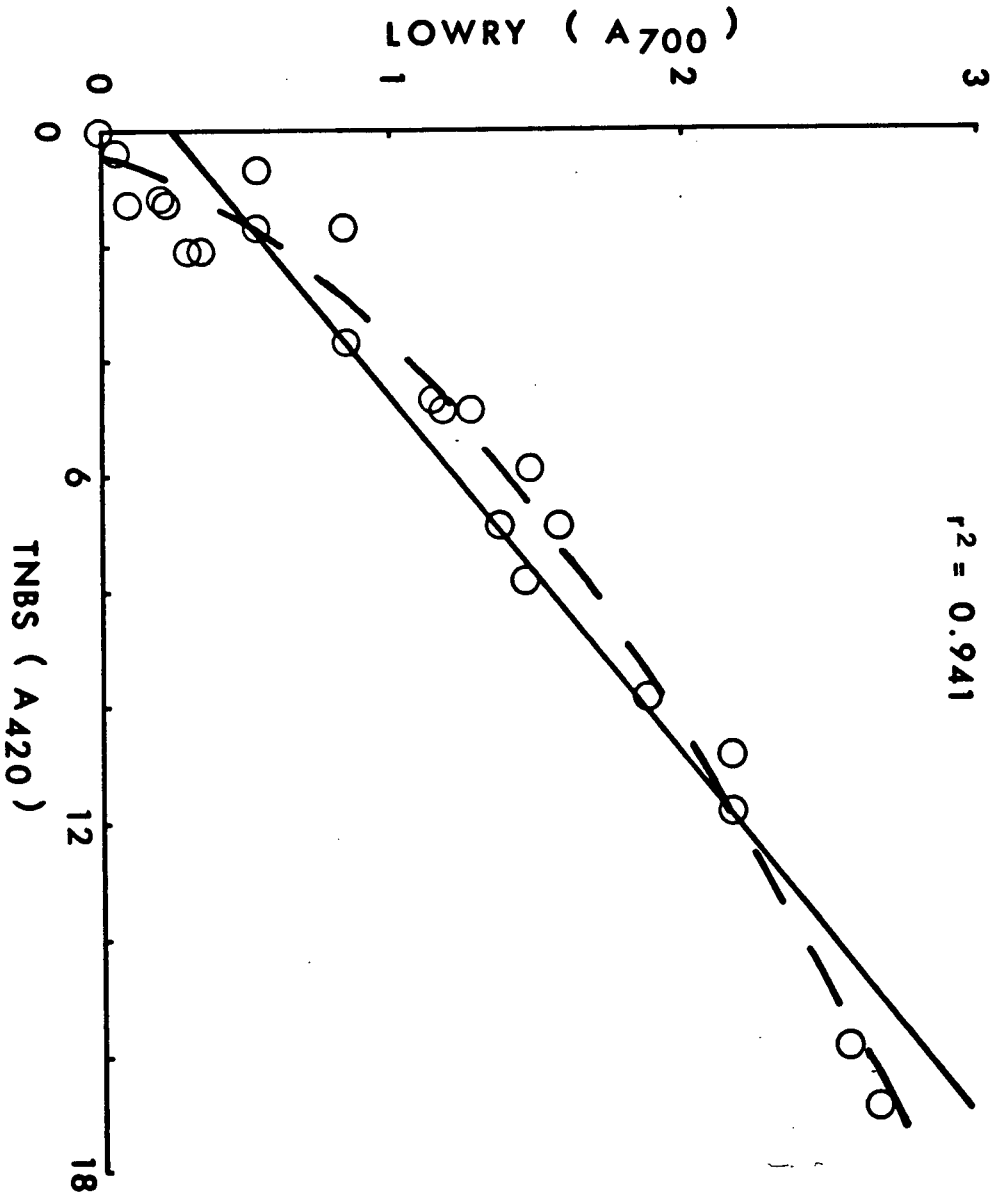

LOWRY (A700)
0
1
2
3
0
6
12
18
TNBS (A420)
r² = 0.941

Fig. 5. Comparison of the TNBS method with the A_{280} method as a measure of protein hydrolysis by Deming's method. Absorbance is expressed as the apparent absorbance of the undiluted solution. The broken line represents the transformed data from the A_{280} method vs. the TNBS method.

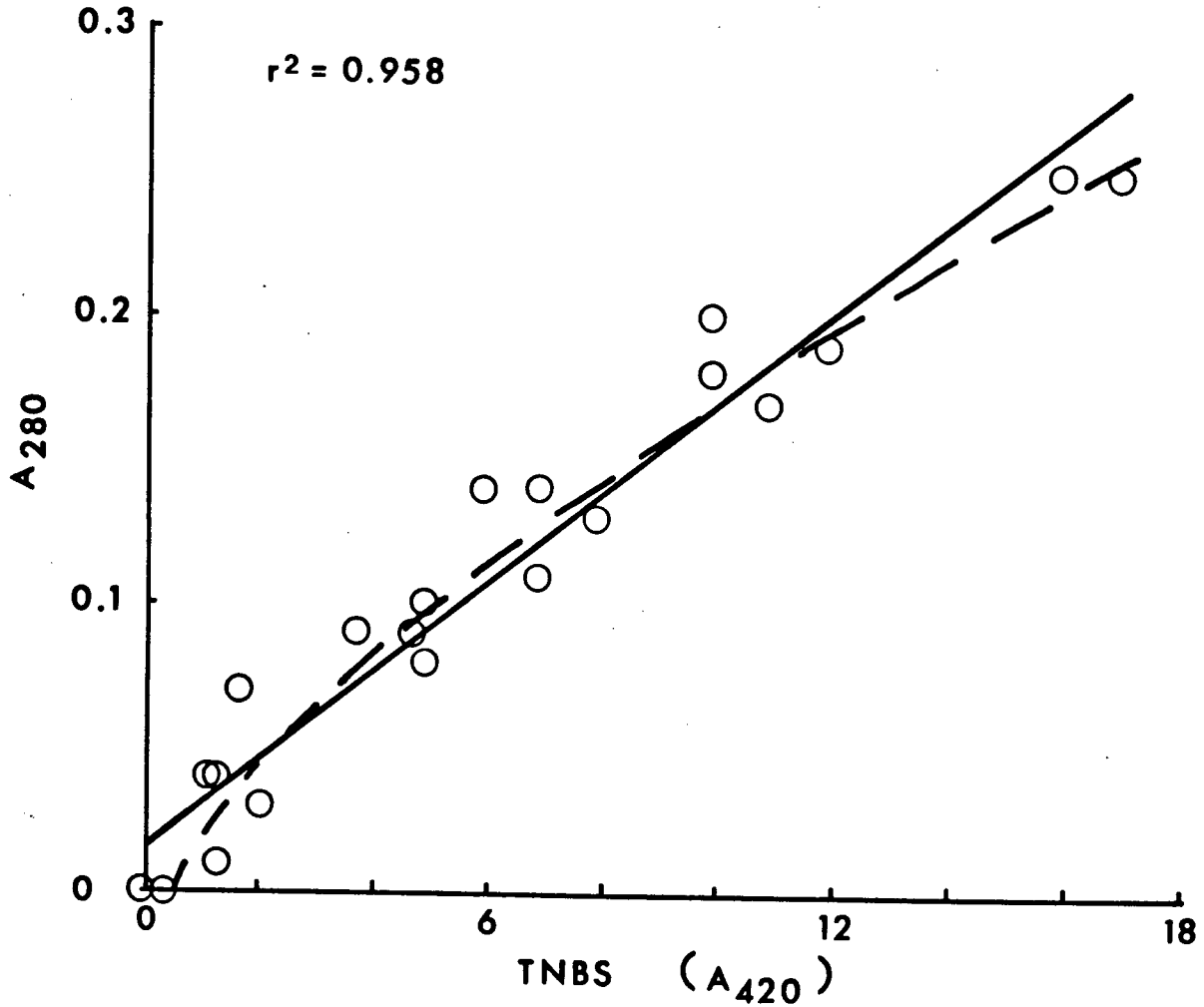

0.3
0.2
0.1
0
$r^2 = 0.958$
A_{280}
0
6
12
18
TNBS (A_{420})

Fig. 6. Comparison of the Lowry method with the A_{280} method as a measure of protein hydrolysis by Deming's method. Absorbance is expressed as the apparent absorbance of the undiluted solution.

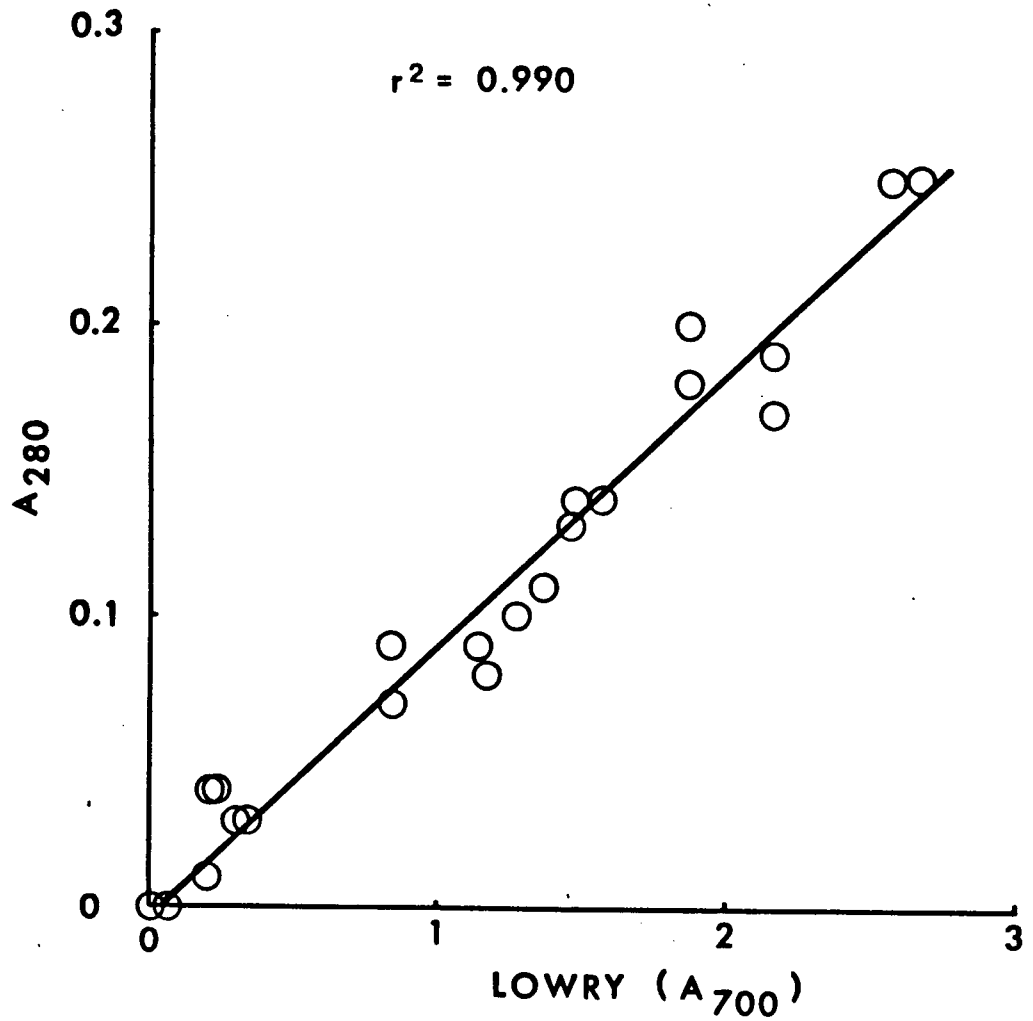

0.3
0.2
0.1
0
r^2 = 0.990
A_{280}
0
1
2
3
LOWRY (A_{700})

Table 1. Detectability of the fluorescamine method (Fluo), the TNBS method, the Lowry method and the absorbance at 280 nm (A_{280}) as estimated by Deming's procedure.

	Fluo	TNBS	Lowry	A_{280}
Direct comparison				
Fluo and TNBS	98 (1.0)	155 (1.58)		
Fluo and Lowry	46 (1.0)		192 (4.17)	
Fluo and A_{280}	72 (1.0)			189 (2.63)
TNBS and Lowry		0.45 (1.58)	1.22 (4.28)	
TNBS and A_{280}		0.64 (1.58)		1.01 (2.49)
Lowry and A_{280}			0.14 (4.17)	0.08 (2.38)
(average)	(1.0)	(1.6)	(4.2)	(2.5)
After linearization				
Fluo and Lowry	47 (1.0)		132 (2.81)	
Fluo and A_{280}	64 (1.0)			158 (2.47)
TNBS and Lowry		0.37 (1.73)	0.60 (2.81)	
TNBS and A_{280}		0.45 (1.28)		0.87 (2.47)
(average)	(1.0)	(1.5)	(2.8)	(2.5)

The figures in parentheses are calculated for direct comparison of detectability by assigning 1.0 to the fluorescamine method.

absorbance at 280 nm was more reliable than the Lowry method.

Schwerdtfeger (1977) claimed that the TNBS method (Schwerdtfeger, 1974) could detect trypsin (Merck) activity at a level as low as 3 ng/ml. However, using the same analytical conditions but with a trypsin from a different source, trypsin activity was detected a level of 260 ng/ml (P = 0.005%; n = 4). This difference in sensitivity may be caused by the difference in the purity of the trypsins used. In comparison, the fluorescamine method proposed could detect trypsin activity at a concentration of 35 ng/ml (P = 0.003%; n = 4) according to the conditions specified by Schwerdtfeger (1974).

Calculation of the degree of dilution of TCA filtrate prior to measurement of absorbance and fluorescence intensity yielded values of 2.00, 1.82, 0.48, 0.28 ml of TCA filtrate per 10 ml final diluted volume for the A_{280}, Lowry, TNBS and fluorescamine methods, respectively. This means the fluorescamine method needs the smallest amount of sample for the best precision.

As indicated already, the two methods for comparing different proteolysis assay procedures yielded some different results. When the detection limit of Schwerdtfeger (1977) was used, the fluorescamine method was 7.4 times (260/35) more sensitive than the TNBS method. On the other hand, the comparison of detectability used in this study showed that the fluorescamine method was 1.5 times more sensitive than the TNBS method. This difference in the sensitivity may be due to the difference in the incubation conditions (24 h at 35 C vs. 7 min at 40 C) and different parameters used (detection limit for the

method of Schwerdtfeger vs. detectability employed in this study).

The four methods chosen were those that could be used in most laboratories and which have been used by various research groups to detect proteolysis in milk and milk products. The microfluorometric method (Castell et al., 1979) was chosen over other published fluorometric methods because less fluorescamine was used in this method. Stein et al. (1974) studied the influence of various parameters on the rate of the reaction of fluorescamine with primary amines and on the rate of hydrolysis of the reagent. It was shown that both were dependent on the reaction conditions, including pH, solvent in which the reagent was prepared, temperature, reagent concentration, and buffer salt. Hence, phosphate buffer was chosen to raise the pH of the TCA filtrate to 8.5 because it was shown to be better than borate buffer for this purpose.

Comparison of methods for estimation of protein hydrolysis is difficult. First, the compounds being analysed are different; aromatic groups (A_{280}, Lowry) vs. amino groups (fluorescamine, TNBS). Second, the instruments used are different; spectrofluorometer (fluorescamine) vs. spectrophotometer (A_{280}, Lowry, TNBS). When the working principle of the instruments is different, direct comparison of the increase in fluorescence intensity and the increase in absorbance is not appropriate (Spencer and Spencer, 1974). Since there is no error-free or referee method, direct application of regression analysis to compare the result of one

method vs. the result of another method is not totally justified.

Deming's method minimizes the weighted sum of squares:

$$S = \Sigma[(x_i - \hat{X}_i)^2 + (y_i - \hat{Y}_i)^2 \lambda] \qquad (1)$$

instead of

$$S = \Sigma[(x_i - \hat{X}_i)^2 + (y_i - \hat{Y}_i)^2] \qquad (2)$$

in the classical linear regression procedure, where $\hat{X}_i$ and $\hat{Y}_i$ be the estimate for X_i and Y_i; λ is a 'weight':

$$\lambda = V(\epsilon) \; / \; V(\delta) \qquad (3)$$

where $V(\epsilon)$ and $V(\delta)$ are the variances corresponding to x and y. Therefore, Deming's method is a weighted linear regression analysis.

The "detectability" described in this study was similar to the sensitivity ratio discussed by Mandel (1964). It was expressed as the ratio of $\sigma_\varepsilon \; / \; \sigma_\delta$ to $| \, dM / dN \, |$, where σ_ε and σ_δ were standard deviations of methods M and N; $| \, dM / dN \, |$ was the slope of the line yielded by the plot of M vs. N. As the sentivity ratio increased, the technical merit of method M decreased with respect to method N. The comparison of the two methods is therefore the comparison of their precisions. However, other measures of precisions, such as standard deviation and coefficient of variation, cannot be used directly for comparing analytical methods. This is because the sentivity ratio is the only one that enjoys the property of invariance with respect to transformation of scale.

In the comparison method suggested in this study, both the standard error of estimate and the slope ratio of a particular

assay method are expressed by either absorbance or fluorescence intensity units. The detectability (ratio of standard error of estimate to slope ratio) parameter cancels out difference in instrument as well as difference in compound analysed.

CONCLUSIONS

The method of Schwerdtfeger (1977) has an advantage, by which the detection limit can be expressed as the minimum amount of enzymes that can be detected. Accordingly, a long incubation time is required for maximizing the sensitivity of assays. However, a drawback of this method is limited data points for statistical computation. To increase the number of data points, many more different concentrations of enzymes should be used for assay.

The method proposed in this study is convenient for comparison since more reliable data can be obtained. This is achieved by changing the incubation time of the same reaction mixture using one enzyme concentration. Nevertheless, the data obtained are nondimensional and thus difficult to convert to more realistic parameters, for example amount of amino acids or enzymes.

Considering these situations, an appropriate method should be chosen or, if necessary both methods should be employed to draw the most reasonable conclusion.

REFERENCES

Adams, D.M., Barach, J.T., and Speck, M.L. 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci. 58:828.

Adams, D.M., Barach, J.T., and Speck, M.L. 1976. Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. J. Dairy Sci. 59:823.

Alichanidis, E. and Andrews, A.T. 1977. Some properties of the extracellular proteases produced by the psychrotrophic bacterium Pseudomonas fluorescens strain AR-11. Biochim. Biophys. Acta 485:424.

Beeby, R. 1980. The use of fluorescamine at pH 6.0 to follow the action of chymosin on κ-casein and to estimate this protein in milk. N. Z. J. Dairy Sci. Technol. 15:99.

Castell, J.V., Cervera, M., and Marco, R. 1979. A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A reexamination of the conditions of reaction. Anal. Biochem. 99:379.

Chism, G.W., Huang, A.E., and Marshall, J.A. 1979. Sensitive assay for proteases in sterile milk. J. Dairy Sci. 62: 1798.

Cogan, T.M. 1977. A review of heat resistant lipases and proteinases and the quality of dairy products. Ir. J. Food Sci. Technol. 1:95.

Cousin, M.A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. J. Food Prot. 45:172.

Fujii, S. and Nakai, S. 1980. Optimization of data transformations for linearization. Can. Inst. Food Sci. Technol. J. 13:188.

Gebre-Egziabher, A., Humbert, E.S., and Blankenagel, G. 1980. Heat-stable proteases from psychrotrophs in milk.

J. Food Prot. 43:197.

Griffiths, M.W., Phillips, J.D., and Muir, D.D. 1981. Thermostability of proteases and lipases from a number of species of psychrotrophic bacteria of dairy origin. J. Appl. Bacteriol. 50:289.

Hull, M.E. 1947. Colorimetric determination of partial hydrolysis of the proteins in milk. J. Dairy Sci. 30:881.

Juffs, H.S. 1973. Proteolysis detection in milk. I. Interpretation of tyrosine value data for raw milk supplies in relation to natural variation, bacterial counts and other factors. J. Dairy Res. 40:371.

Juffs, H.S. 1975. Proteolysis detection in milk. IV. Starch-gel electrophoresis and formol titration. J. Dairy Res. 42:277.

Kas, J. and Rauch, P. 1982. Radioisotopic method for the determination of low and very low proteolytic activities in foodstuffs. Z. Lebensm. Unters. Forsch. 174:290.

Koops, J., Klomp, H., and Elgersma, R.H.C. 1975. Rapid determination of nitrogen in milk and dairy products by colorimetric estimation of ammonia following an accelerated digestion procedure. Neth. Milk Dairy J. 29:169.

Law, B.A. 1979. Reviews of the progress of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. 46:573.

Law, B.A., Andrews, A.T., Cliffe, A.J., Sharpe, M.E., and Chapman, H.R. 1979. Effect of proteolytic raw milk psychrotrophs on Cheddar cheese making with stored milk. J. Dairy Res. 46:497.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

Mandel, J. 1964. In The Statistical Analysis of Experimental Data. John Wiley and Sons, Inc., New York. pp 366-369.

McKellar, R.C. 1981. Development of off-flavor in ultra-high temperature and pasteurized milk as a function of proteolysis. J. Dairy Sci. 64:2138.

Nakai, N., Lai, C.Y., and Horecker, B.L. 1974. Use of fluorescamine in the chromatographic analysis of peptides from proteins. Anal. Biochem. 58:563.

Pearce, K.N. 1979. Use of fluorescamine to determine the rate of release of the caseino-macropeptide in rennet-treated milk. N. Z. J. Dairy Sci. Technol. 14:233.

Richardson, B.C. and Te Whaiti, I.E. 1978. Partial characterization of heat-stable extracellular proteases of some psychrotrophic bacteria from raw milk. N. Z. J. Dairy Sci. Technol. 14:172.

Richter, R.L., Schmidt, R.H., Smith, K.L., Mull, L.E., and Henry, S.L. 1979. Proteolytic activity in ultra-pasteurized aseptically packaged whipping cream. J. Food Prot. 42:43.

Schwabe, C. 1973. A fluorescent assay for proteolytic enzymes. Anal. Biochem. 53:484.

Schwerdtfeger, E. 1974. Automatisch-kinetisch Bestimmung von pflanzlichen Enzymaktivitaten und Inhaltstoffen. 2. Bestimmung von Aminosauren. Z. Lebensm. Unters.-Forsch. 156:266.

Schwerdtfeger, E. 1977. Nachweisgrenze proteolytischer Aktivitaten. Mikrochimica Acta II:363.

Snoeren, T.H.M., van der Speck, C.A., Dekker, R., and Both, P. 1979. Proteolysis during the storage of UHT-sterilized whole milk. I. Experiments with milk heated by the direct system for 4 sec at 142 C. Neth. Milk Dairy J. 33:31.

Snoeren, T.H.M., and Both, P. 1981. Proteolysis during the storage of UHT-sterilized whole milk. II. Experiments with milk heated by the indirect system for 4 sec at 142 C. Neth. Milk Dairy J. 35:113

Spencer, P.W. and Spencer, R.D. 1974. Globulin-specific proteolytic activity in germinating pumpkin seeds as detected by a fluorescence assay method. Plant Physiol. 54:925.

Stein, S., Bohlen, P., and Udenfriend, S. 1974. Studies on the kinetics of hydrolysis of fluorescamine. Arch. Biochem. Biophys. 163:400.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., and Weigele, M. 1972. Fluorescamine: A reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science 178:871.

Wakkers, P.J.M., Hellendorn, H.B.A., Op de Weegh, G.J., and Heerspink, W. 1975. Applications of statistics in clinical chemistry. A critical evaluation of regression lines. Clin. Chim. Acta 64:173.

CHAPTER 2

Identification and Study of Proteolytic Pseudomonads Isolated from Raw Milk

INTRODUCTION

Pseudomonads are the predominant species of the psychrotrophic microflora of refrigerated raw milk (Thomas and Thomas, 1973; Tompkin, 1973; Stadhouders, 1975). Many of these species produce extracellular enzymes which can degrade both milk proteins and milk fat (Law, 1979; Muir et al., 1979). Even though the effects of these pseudomonads on milk and milk products have been extensively studied, there is little information on the phenotypic characteristics of these species.

Identification of these pseudomonads according to the Bergey's Manual of Determinative Bacteriology (1974) is commonly employed (Fox et al., 1976; Cousin and Marth, 1977; Richardson and Te Whaiti, 1978; Gebre-Egziabher et al., 1980a). In most of these studies, the bacterial isolates were identified to the genus level. The characteristics studied, from which identification was made, were often omitted in published papers. It is difficult for the reader to assess the validity of the identification. If the number of characters studied is small, as in identifying to the genus level, a perfect match with those named microorganisms in an established classification will be common. If a large number of characters are studied, as in identifying to the species level, then a perfect match will seldom be possible. One has to go with a near match in which the number of divergencies is small. The inclusion of a certain species into a named taxon will very much depend on the choice of characters and methods employed.

At present, there is not enough information, at the species level, about pseudomonads in raw milk. The object of this research was to identify to the species level, by numerical taxonomy, proteolytic pseudomonads isolated from raw milk. It was hoped that these pseudomonads could be classified into separate groups. Representative isolates could then be selected in the future for more detailed studies of their biochemical activities relevant to spoilage. Identification by using diagnostic tables is of limited value because of the difficulty of identifying strains aberrant in one or more of the characters studied. Hence, a numerical taxonomy approach to identification using cluster analysis was chosen instead. Growth and proteinase activity of eight proteolytic pseudomonads were also examined. This served to determine if there was any relationship between proteinase activity in milk and proteolytic psychrotrophic counts.

LITERATURE REVIEW

A. Pseudomonas species in raw milk

The isolation of Pseudomonas species in raw milk has been well documented (Witter, 1961; Thomas and Thomas, 1973; Stadhouders, 1975; Cousin, 1982). In their study on the thermostability of proteases from psychrotrophic bacteria of dairy origin, Griffiths et al. (1981) reported that 53% of the psychrotrophic population were Pseudomonas species. Species of the genus Serratia contributed only 16% of the microflora. Muir et al. (1979) concluded that Pseudomonas was the predominant genus in raw milk collected over a period of 14 months. Pseudomonads accounted for 65% - 78% of the psychrotrophic population. They found that there was no seasonal pattern in frequency of isolation of Pseudomonas groups. Species of the Enterobacteriaceae family were found more frequently in the summer months of May, June and July. Juffs (1973) studied the microflora of milk produced in South Eastern Queensland, Australia. Of the 330 isolates studied, 167 were identified as Pseudomonas species. Samagh and Cunningham (1972) reported that 28% of the psychrotrophic microflora belonged to the genus Pseudomonas. Audrey and Frazier (1959) obtained 17% Pseudomonas species out of 220 psychrotrophic microorganisms isolated from raw milk collected from 12 farms.

B. Approaches to identification

Pseudomonas fluorescens is considered by numerous

researchers to be the predominant species contaminating raw milk (Cousin, 1982). Identification of the bacterial isolates is most commonly performed according to the Bergey's Manual of Determinative Bacteriology (1974) or the diagnostic tables of Cowan and Steel (1965) (Fox et al., 1976; Cousin and Marth, 1977).

1) Identification according to the Bergey's Manual of Determinative Bacteriology (1974).

According to Bergey's Manual, members of the genus Pseudomonas are motile, strictly aerobic, Gram-negative rods which are catalase and oxidase positive. Most species require no growth factors. Diffusible fluorescent pigments are produced by some species. Detailed characteristics of 29 species, incubated at 30 C, are also given. For P. fluorescens, 14 principal distinctive characteristics are tabulated and other phenotypic characteristics are also described.

Keys are given for the primary separation of species into groups that can be handled conveniently. The key to identify P. fluorescens is given as: growth factors are not required, poly-β-hydroxybutyrate is not accumulated, fluorescent pigments are produced and the arginine dihydrolase system is present. It also states that even important differential traits may be absent in some strains when isolated, or may be lost after laboratory cultivation. To avoid the risk of misidentification of a strain aberrant in one or more of the differential traits, the diagnostic keys should not be solely relied upon.

Characters given in the table and description for that suspected species should also be studied.

Tayfour et al. (1982) identified a psychrotrophic strain as P. fluorescens biotype A according to the Bergey's Manual of Determinative Bacteriology (1974). Information on how the identification was made was not given. Richardson (1981) purified and characterized a heat-stable protease from P. fluorescens isolated from refrigerated raw milk. No detailed description of the characters studied were provided. Law et al. (1979a) identified five proteolytic Gram-negative isolates from raw milk as P. fluorescens according to the schemes outlined in Bergey's Manual.

These studies have one common mistake: there is insufficient information on how the identification was done. It would be more reassuring if the characters studied and the incubation temperature employed were included.

2) Identification according to the Manual for the Identification of Medical Bacteria (Cowan and Steel, 1965)

In this manual, Pseudomonas are defined as Gram-negative, motile, aerobic rods which are catalase and oxidase positive. Diffusible yellow or green pigments may be produced by members of this genus. A diagnostic table of characteristics for only three Pseudomonas species, incubated at 37 C, was also included. The three species described are P. aeruginosa, P. fluorescens and P. pseudomallei. Of the three species, only P. fluorescens can grow at 5 C but not at 42 C. Obviously, species other than

the three species listed can not be identified according to this manual. This manual can only be consulted if the purpose is to identify the milk isolates to the genus level.

Muir et al. (1979) isolated psychrotrophic bacteria from supplies of raw milk. The Gram-negative bacterial isolates were grouped by the first-stage diagnostic procedure of Cowan and Steel (1965). They found that species of the genus Pseudomonas were the predominant psychrotrophic bacteria in raw milk. Cousin and Marth (1977) examined changes in milk proteins caused by psychrotrophic bacteria. Five of the nine bacterial isolates examined were Pseudomonas species. The pseudomonads were identified according to the diagnostic tables of Cowan and Steel (1965) and Bergey's Manual (1974). Modification of milk proteins by Pseudomonas species were also investigated by DeBeukelar et al. (1977). Isolates were classified into genera by the diagnostic tables of Cowan and Steel (1965) and Breed et al. (1957).

3) Identification by the API 20E system (Analytab Products, Plainview, NY)

The use of the API 20E system to identify bacterial isolates from raw milk has been reported (McKellar, 1981). He studied the relationship between proteolysis and off-flavor development in UHT milk caused by three bacterial isolates identified to be strains of Pseudomonas. He used an incubation temperature of 22 C instead of 35 C as recommended. Additional information on the ability of the isolates was also included.

Griffiths et al. (1981) characterized the Gram-negative rod-shaped bacteria, isolated from raw milk, using API 20E strips incubated at 30 C. The majority of the strains reported were P. putida, P. fluorescens and P. fragi.

The API 20E system is a ready-to-use, microtube system designed for the performance of 23 biochemical tests. This system is designed mainly for the identification of Enterobacteriaceae. The data base is based on results after 18 - 24 hours of incubation at 35 - 37 C. The application of this system to identify bacterial cultures not of clinical origin has yet to be established. The accuracy of this system to identify nonfermentative bacilli is beginning to gain attention (Otto and Blachman, 1979). Results obtained from using this system, especially with an incubation temperature other than 35 - 37 C, should be evaluated with care.

4) Identification by cluster analysis

The weakness of current classifications is that genera and species are still described on the basis of one or a few specific characters. Production of fluorescent pigment, accumulation of poly-β-hydroxybutyrate and the presence of the arginine dihydrolase system are a few required characters for a strain to possess to be identified as Pseudomonas. Such classifications carry the risk of misidentification, for a strain may be aberrant in one of the specific characters used to make a classification.

In order to accomodate strain variation, identification

using cluster analysis is used. The bacterial isolates are identified on the basis of a large set of characters, with each character being given equal weight. The similarity of characteristics between pairs of organisms is expressed numerically, usually in terms of a simple matching coefficient. Those bacterial isolates with high similarity values are arranged together to form clusters or groups.

There are three types of cluster analysis: the single-linkage, complete-linkage and average-linkage. With the average-linkage clustering methods, the unweighted arithmetic mean analysis is the most common. The three methods differ in the criterion for admission of a bacterial isolate to a cluster or for fusion of clusters. In complete-linkage analysis, an isolate is not admitted to a cluster until its simple matching coefficients with all members of the cluster are equal to or greater than the level set for cluster formation, and two clusters can fuse only when the simple matching coefficients between all isolates in the two clusters are equal to or greater than the set clustering level. In single-linkage analysis, linkage between an isolate and any member of a cluster at a given similarity level provides admission to that cluster, and two clusters can fuse with linkage between any pair of isolates (one from each cluster). In average-linkage analysis, an isolate is not admitted to a cluster until the mean of its simple matching coefficients with all members of the cluster are equal to or greater than the level set for cluster formation, and two clusters can fuse only when the mean of all the

similarity coefficients between all isolates in the two clusters are equal to or greater than the set clustering level.

Juffs (1973), using complete-linkage cluster analysis, identified Pseudomonas species isolated from raw milk. Relevant characters of 13 Pseudomonas species as proposed by Stanier et al. (1966) were also incorporated in the analysis. Twenty nine biochemical characters of 180 bacterial isolates were examined. All characterizations were performed at 28 C. The dendrogram obtained by cluster analysis showed that P. fluorescens was the predominant species. The clusters, in which the majority of the milk isolates resided in, were quite distinct from the reference species proposed by Stanier et al. (1966). Samagh and Cunningham (1972) used single-linkage cluster analysis to identify 182 pseudomonads from milk. Sixteen known representatives of different Pseudomonas species, along with the 182 milk isolates were examined for 97 characters at 20 C. Their results indicated that P. fluorescens, P. aeruginosa-like organisms and P. putida were the major species. They also suspected that 41 nonfluorescent cultures, which they did not include in the analysis, were related to P. fragi or P. putrefaciens.

5) Identification with no references provided

Richardson and Newstead (1979) reported the effects of heat-stable proteases, from two P. fluorescens strains, on the storage life of ultra-high temperature treated milk. The approach used to identify these two strains was not provided.

Juffs (1974) isolated three strains of P. fluorescens and three strains of P. aeruginosa from raw milk. The identification scheme employed was not published. Interestingly, Adams et al. (1975) defined isolates belonging to the genus Pseudomonas as motile, aerobic, Gram-negative rods that grew at 32 C but not at 37 C. The reference to support this classification was not reported.

6) Rapid characterization of bacteria by using a multipoint inoculator

Beech et al. (1955) designed a multipoint inoculator for plating bacteria rapidly. The device consisted of 24 vertical stainless steel rods attached to the inner basal surface of an inverted stainless steel cup. Inoculum (1 ml) was held in 9 x 50 mm tubes positioned by an inoculum tube holder. The holder was a circular plate drilled with 24 holes, in the same pattern as the inoculating rods, each capable of holding a 9 x 50 mm tube. The inoculator was dipped into the inoculum tubes, and then withdrawn and transferred to a nutrient agar plate to achieve a multiple inoculation. In this way, a plate would be simultaneously inoculated with 24 microorganisms in 30 seconds as compared with 8 minutes required for inoculating them singly with a wire loop. A similar multiple inoculation device was reported by Smith (1961). The inoculator was made of a circular steel plate , 8.5 cm in diameter, fitted on the upper side with a centrally placed handle of stainless steel rod. Into each of symmetrically arranged holes bored in the plate was fitted a 1.5

cm stainless steel bolt. These bolts constituted the inoculation stubs. The container for inocula consisted of 8 cm diameter discs of plexiglass sheet drilled to give 16 rounded wells, concentrically aligned with the multiple inoculator stubs. About 0.3 ml of bacterial suspension to be tested were pipetted into each of the wells. Inoculation was done by immersing the inoculator into the wells followed by transfer from the wells to appropriate agar plates. Kaneko et al. (1977) developed a multiple syringe inoculator for agar plates. The apparatus was not simple as it consisted of 5 components: an inoculator holder; the front plate; the syringe holders; the syringe pressure plate; and the syringe inoculators. Inoculation was quite different from the two methods discussed as the plate had to be in the inverted position during inoculation.

Fung and Hartman (1972) developed a simple multiple inoculation device capable of making 96 inoculations at a time. It was made by inserting 27 mm pins separately into 96 wax-filled wells (8 x 12 configuration) of a tissue culture plate. A culture plate was prepared by introducing 0.20 ml of a different broth culture into each of the wells of a sterile tissue culture plate. Sterilization of the inoculating device was achieved by dipping the pins into 90% alcohol for 20-30 s followed by flaming. The sterile inoculation device was charged by lowering it into the culture plate. The charged inoculation device was placed on a dried surface of appropriate agar.

The microtiter technique of Fung and Hartman (1972) was

used by Casas et al. (1977) to enumerate mesophiles, psychrotrophs and coliforms in raw and pasteurized milk. They reported that the Microtiter Count method was reliable when compared with the Standard Plate Count method. The microtiter technique was also used to identify, to the genus level, bacterial cultures isolated from raw milk (Otte et al., 1979).

MATERIALS AND METHODS

A. Isolation of proteolytic strains from raw milk

Proteolytic psychrotrophs in raw milk were isolated on skim milk agar (4 C, 7 d) (Lee, 1976). Isolated colonies exhibiting zones of clearing were purified on skim milk agar incubated at 22 C. They were then stored at 4 C on nutrient agar (Difco, MI) slants as stock cultures with subculture at 8 - 10 week intervals. Type strains of four Pseudomonas species, P. fluorescens ATCC 948; P. fluorescens biotype A ATCC 17397; P. putida ATCC 12633 and P. fragi ATCC 4976) were included for reference purposes.

Each isolate was grown in nutrient broth (Difco, MI) for 24 h at 22 C before characterization. All tests were incubated at 22 C except when specified.

B. Primary characterization tests

1) Gram stain

The Hucker's modification was used (Cowan and Steel, 1974).

2) Oxidase reaction

The method of Kovacs (1956) was used. The test organism was picked with a platinum wire and smeared across the surface of a piece of filter paper impregnated with 2 to 3 drops of 1%

tetramethyl-p-phenyldiamine dihydrochloride paper. A positive reaction was shown by the development of a dark purple color within 10 s.

3) Catalase test

Isolates were scraped from the skim milk agar and suspended in a drop of 3% H_2O_2 on a slide. Evolution of bubbles within one minute was recorded as positive.

4) Growth at pH 4.5

Brain heart infusion broth (Difco, MI) was inoculated with one loopful of culture grown for 24 h in nutrient broth. Cultures were examined for growth after 7 d incubation.

5) Oxidation or fermentation of glucose

The Hugh and Leifson (1953) method was employed. Mineral oil, about 1 cm in depth, was used to seal the tube. A strict aerobe caused the development of a yellow color in the open tube and no change in color in the sealed tube.

6) Motility

Motility was examined by both the hanging drop technique and motility medium (Cowan and Steel, 1974).

Motile organism migrated through the medium which became turbid. Growth of non-motile organism was confined to the stab line.

7) Growth on Pseudomonas isolation agar

The ability of each milk isolate to grow on Pseudomonas isolation agar (Difco, MI) was examined.

Pseudomonas species were defined as Gram-negative, motile rods. They were strict aerobes and could not grow in brain heart infusion broth at pH 4.5. They were oxidase and catalase positive (Stanier et al., 1966; Hugh, 1973).

C. Secondary characterization tests

1) Growth at 4 C and 41 C

Ability of isolates to grow on skim milk agar at 4 C (7 - 10 d) and 41 C (2 d) was evaluated.

2) Production of fluorescent pigments

Medium B of King et al. (1954) was used. Fluorescence was confirmed under ultraviolet light (350 nm).

3) Nitrate reduction

Following incubation of each isolate in nitrate broth (Difco, MI) for 5 d, nitrite was detected with sulphanilic acid and α-naphthylamine. The presence of a red color indicated that nitrate had been reduced to nitrite. If no color development was recorded, zinc dust was added to determine the presence of nitrate not reduced by the test organism. A red color showed that nitrate was not reduced, for the zinc dust reduced the

nitrate to nitrite. The absence of a red color, after the addition of zinc dust, showed that nitrate had been reduced but not to nitrite.

4) Arginine dihydrolase

The method of Niven et al. (1942) was used. The test organism was incubated in arginine broth for 24 h. Arginine hydrolysis was indicated by the development of a brown color after the addition of the Nessler's reagent.

5) Indole production

Indole production from 1% tryptone broth (Difco, MI) after 2 d was recorded as positive by the presence of a red color after addition of Kovacs' reagent.

6) Methyl red

The method of Clarke and Lubs (1915) was used. Methyl red solution was added to an inoculated methyl red-Voges-Proskauer (MR-VP) broth (BBL Microbiology Systems, MD) after incubation for 4 d. A positive reaction was indicated by the development of a red color.

7) Voges Proskauer

The method of Barritt (1936) was followed. To a 48 h culture grown in MR-VP medium, 5% α-naphthol solution followed by 40% potassium hydroxide solution were added. A positive reaction was indicated by a strong red color.

8) Tween 80 hydrolysis

The method as described in Cowan and Steel (1974) was adopted. For up to 7 days, plates were examined for the presence of an opaque halo around isolated colonies.

9) Butterfat hydrolysis

The medium described by Jones and Richards (1952) was followed. Lipolysis was recorded as positive when a distinct blue zone was seen around the colony within 7 d.

10) Phospholipase production

Lecithin medium (Chrisope et al., 1976) was used. Plates were examined, over a period of 7 days, for the presence of colonies surrounded by an opaque zone.

11) Gelatinase production

The method according to Kohn (1953) using charcoal gelatin disc (Oxoid, Ottawa, Canada) was followed. Hydrolysis was shown by the appearance of free charcoal particles in the peptone solution (0.1% w/v).

12) Proteinase production

Skim milk agar was employed and a clear zone around colonies, after flooding with 10% acetic acid, was taken as an indication of proteinase production.

13) Accumulation of poly-β -hydroxybutyrate

The ability to accumulate poly-β-hydroxybutyrate using the medium recommended by Stanier et al. (1966) was evaluated. The polymer was detected by microscopic examination of bacteria after staining with Sudan Black B (Sigma, St. Louis, MO).

14) Utilization of the different carbon sources

The ability to grow on 35 compounds as the sole source of carbon was studied. The basal medium was that of Dowson (1949). All compounds were filter sterilized before addition to the autoclaved medium. The substrate concentration was 0.1% (w/v) except for sugars (0.2%) and phenol (0.025%).

The sugars tested were D-ribose, D-xylose, L-arabinose, L-rhamnose, D-glucose, D-mannose, D-galactose, D-fructose, sucrose, trehalose, maltose, lactose, gluconate and salicin. Other compounds tested included acetate, propionate, butyrate, succinate, malate, lactate, citrate, pyruvate, mannitol, inositol, adonitol, glycerol, propyleneglycol, ethanol, methanol, glycine, L-alanine, L-serine, L-valine, L-arginine.

15) Utilization of the different nitrogenous compounds

The ability to use acetamide, nicotinate, and panthothenate as sole nitrogen source was tested on the basal medium of Dowson (1949). The substrate concentraion was 0.1% (w/v). The three compounds were filter sterilized.

D. Rapid characterization using a multipoint inoculator

A multipoint inoculator, developed after that of Fung and Hartman (1972), was constructed by fixing 96 stainless steel pins (25 mm long) into a piece of 8.5 cm x 12.5 cm x 0.5 cm thick balsa wood (Fig. 7). The pins were positioned to correspond with the centers of the 96 wells of a tissue culture plate (Falcon, Oxnard, CA). A piece of 9 cm x 13 cm x 1 cm thick high density polyethylene board (Cadillac Plastic, Vancouver, B.C., Canada) was placed on top of the balsa wood. A knob with a 5 cm screw was used to lock the board and the balsa wood in place. The whole system was wrapped with aluminum foil before the pins were inserted. The inoculation device was sterilized by autoclaving (121 C, 15 min).

Cultures grown overnight in nutrient broth (22 C) were introduced into the wells of a 96 well tissue culture plate. Media to be inoculated were dispensed into the wells of another plate. An experimental design whereby different broth cultures were introduced into different rows of wells (12 wells / row) of the 8 x 12 configuration tissue culture plate was employed. Different media were then dispensed into different columns of wells (8 wells / column).

The sterile multipoint inoculator was charged by dipping the pins into the wells of the plate containing the broth cultures. The charged inoculator was then placed on top of a tissue culture plate containing the appropriate media so that all the pins were immersed in the media. The inoculator was then sterilized, by dipping the pins into 90% alcohol followed by flaming.

Fig. 7. The multipoint inoculator.

This rapid characterization method was used to study the ability of the bacteria isolated from milk to use 35 compounds as the sole source of carbon and 3 compounds as the sole source of nitrogen. For the rest of the characterizations, the conventional methods were followed.

E. Numerical taxonomy

Bacterial isolates identified as Pseudomonas together with the 4 reference strains: P. fluorescens ATCC 948; P. fluorescens biotype A ATCC 17397; P. putida ATCC 12633 and P. fragi ATCC 4976 were subjected to numerical taxonomic analysis. In addition, results of the relevant characters studied for the 26 Pseudomonas species as described in the Bergey's Manual of Determinative Bacteriology (1974) were also included. The 26 species included were: P. aeruginosa ; P. putida ; P. fluorescens ; P. chlororaphis ; P. cichorii ; P. stutzeri ; P. mendocina ; P. alcaligenes ; P. pseudoalcaligenes ; P. pseudomallei ; P. mallei ; P. caryophylli ; P. cepacia ; P. marginata ; P. lemoignei ; P. testosteroni ; P. acidovorans ; P. delafieldii ; P. solanacearum ; P. facilis ; P. saccharophila ; P. ruhlandii ; P. flava and P. palleroni . For P. fluorescens , three biotypes were also studied. The species P. maltophilia , P. vesicularis and P. diminuta were not subjected to this analysis because these species require one or more growth factors while no growth factor was required by any of the milk isolates.

The character data were coded in a two-state coding format; a positive (+) reaction was scored as 1, and a negative (-) reaction as 0. A data matrix consisting of 49 bacterial strains scored for 52 characters was obtained. The simple matching coefficients (S_{SM}) between any pair of bacterial strains were computed and the strains were clustered by unweighted pair-group average-linkage analysis (Sneath and Sokal, 1973). The analysis and the printing of the dendrogram were performed on the Michigan Terminal System (MTS) Amdahl 470 V/8 computer at the University of British Columbia with the NT-SYS program (1980) obtained from the State University of New York at Stony Brook.

F. Hydrolytic characteristics

1) Proteinase production at 4 C, 22 C and 32 C

Ability of isolates to produce zones of clearing on skim milk agar at 4 C (7 - 10 d), 22 C (2 d) and 32 C (2 d) were examined by surface plating on skim milk agar followed by incubation at the appropriate temperature.

2) Gelatinase production at 4 C and 22 C

Ability of the isolates to hydrolyze gelatin charcoal disc at 4 C (30 d) and 22 C (14 - 20 d) were evaluated.

Data from butterfat, Tween 80 and lecithin hydrolysis studies (22 C) were also included in this evaluation.

G. Proteolytic pseudomonads incubated in sterile milk

Growth and proteinase activity of eight proteolytic pseudomonads isolated from raw milk were studied. The eight isolates studied were strains 0-0, 2-1, 8-1, 10-0, 2-2, 4-1, 6-3, and 8-2. Prior to culturing, the eight proteolytic isolates were grown separately (22 C, 48h) in nutrient broth. The cultures were then transferred separately to 150 ml sterilized (15 min, 121 C) 10% (w/v) reconstituted skim milk (RSM) to give a final population of 10^3 - 10^4 cfu/ml milk. Growth and enzyme activity were monitored over a 19 day storage period at 4 C with shaking. Samples were withdrawn immediately after inoculation and every 2 or 3 days during the storage period for determination of bacterial counts and proteinase activity. Serial dilutions of milk in 0.1% peptone broth were drop plated on SMA followed by incubation at 4 C for 7 days.

Proteinase activity for each isolate was determined by the fluorescamine method (Castell et al., 1979). On each sampling day, 2 ml of the inoculated milk sample were mixed with 2 ml of 24% (w/v) trichloracetic acid (TCA). Another 10 ml sample were withdrawn and placed in a test tube. It was then incubated at 40 C for 2 h. To prevent further bacterial growth, 0.01 ml of 2% (w/v) sodium azide was added to the milk sample. An incubation temperature of 40 C was employed because it was found to be the optimal temperature for most proteinases from psychrotrophic bacteria isolated from raw milk (Gebre-Egziabher et al., 1980a).

After incubation the reaction was stopped by adding an

equal volume of 24% (w/v) TCA. The TCA-soluble non-protein nitrogen was separated from the precipitated casein by centrifugation (10,000 x g, 5 min). The supernatant was decanted and filtered through Whatman No. 54 filter paper. A 0.1 ml portion of the filtrate was mixed with 0.3 ml 3 M potassium phosphate dibasic followed by the addition of 0.15 ml of 0.03% (w/v) fluorescamine in acetone. The mixture was immediately mixed. To adjust the volume to cuvette size, 3.0 ml of distilled water were added. Relative fluorescence (excitation wavelength = 395 nm; emission wavelength = 480 nm) was determined with an Aminco Bowman spectrofluorometer 4-8202 (American Instrument Co., Silver Spring, MD) with a xenon lamp.

Fluorescence intensities were converted to micromoles of free amino groups per milliliter of milk by a tyrosine standard curve. Proteolysis was defined as the increase in the concentration of TCA-soluble free amino groups per milliliter of milk after incubation at 40 C for 2 h.

H. Electrophoresis

The pH of the 10% RSM samples taken during storage were adjusted to 4.6 with trichloracetic acid buffer (Nakai et al., 1964) to precipitate the caseins. The precipitate was redissolved twice in the buffer and freeze dried. The caseins were saved for electrophoresis.

An Atto vertical gel electrophoresis unit (Atto Corp., Japan) was used. A 7.5% separation gel was prepared by mixing

2.33 g of acrylamide (Sigma, St. Louis, MO) and 0.06 g N,N'-methylene-bis-acrylamide (Bis) (Bio-Rad, Richmond, CA) with 8 ml of water and 4 ml of 0.38 M Tris-HCl buffer (pH 8.9). Polymerization was induced by the addition of 16 ml of 1.07% (w/v) ammonium persulfate (Bio-Rad, Richmond, CA) and 0.03% (w/v) TEMED (Sigma, St. Louis, MO). Urea and 2-mercaptoethanol were added to a final concentration of 7 M and 22 mM. A layer of water was added to promote polymerization.

The spacer gel was prepared by mixing 0.5 g acrylamide and 0.12 g Bis with 5 ml of water and 2 ml of 0.06 M Tris-HCl buffer (pH 6.7). Urea was added to a concentration of 7 M. Polymerization was induced by addition of 3 ml of 0.004% (w/v) riboflavin and 0.06% (w/v) TEMED. A fluorescent light was placed directly behind the electrophoresis cell after a comb, to form the sample slots, was inserted. After 2 h the comb was removed and the slots washed with electrode buffer (0.38 M glycine, 0.05 M Tris pH 8.3) before the samples were applied.

Casein samples (0.5%, w/v) were suspended in pH 6.8 buffer (0.05 M Tris-glycine, 7 M urea, 70 mM 2-mercaptoethanol). Forty microliters of sample were introduced into each sample well.

Electrophoresis was performed with a current of 3 mA/cm of gel for about 4 h. The gel was fixed and stained with Coomassie Blue R-250 (0.05%, w/v; Bio-Rad, Richmond, CA) in methanol:water:acetic acid (50:40:10 v/v) for 1 h. The gels were destained in methanol:water: acetic acid (25:68:7 v/v) for at least 24 h. The gels were dried under vacuum (55 C, 1 h).

RESULTS AND DISCUSSION

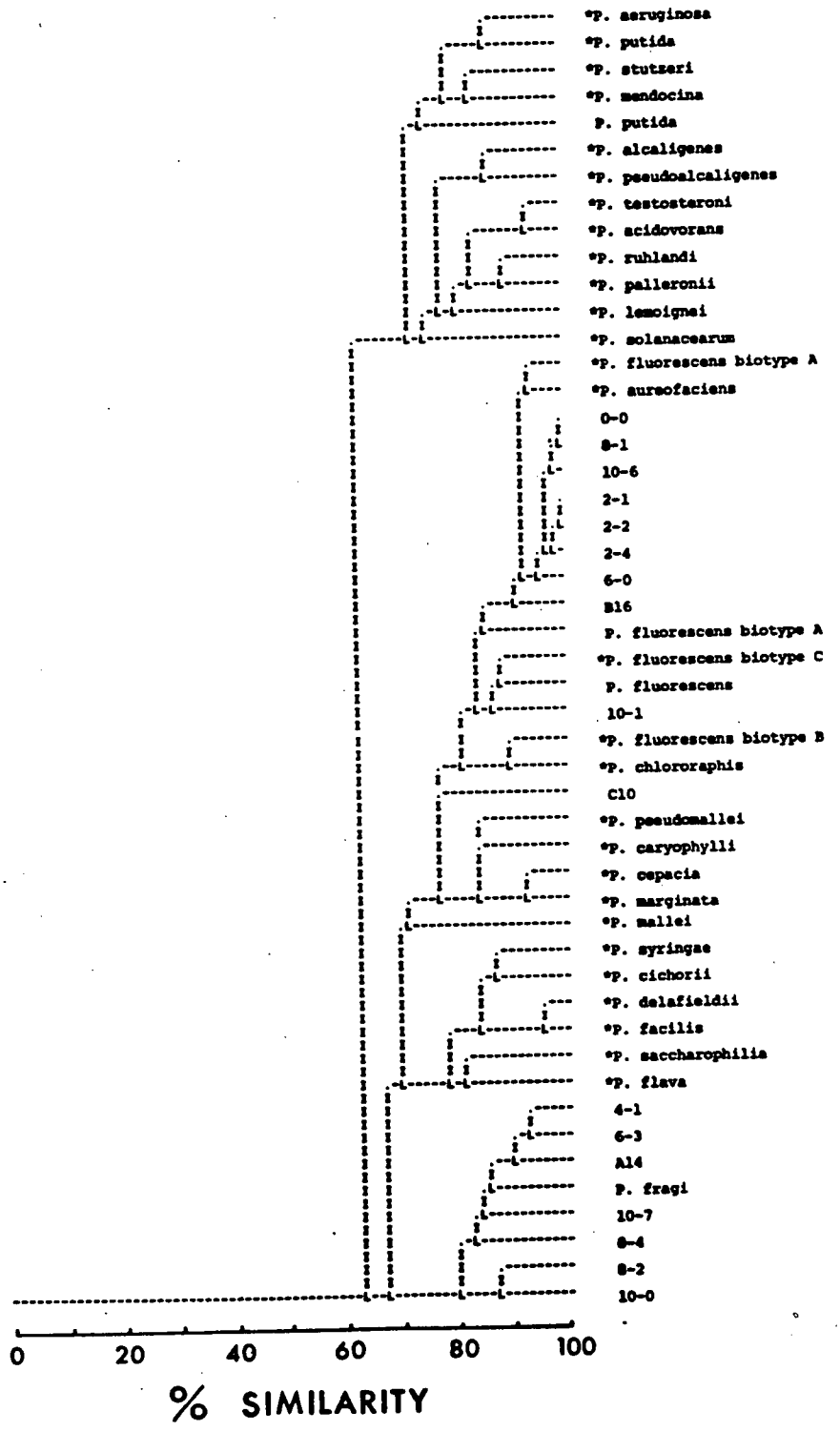
A. Identification of proteolytic pseudomonads in raw milk

The dendrogram constructed from the unweighted pair-group average-linkage cluster analysis is shown in Fig. 8. Since the Bergey's Manual of Determinative Bacteriology (1974) used a monothetic classification of the 29 *Pseudomonas* species, no attempt was made to compare that classification with the one shown in Fig. 8. Emphasis was put on the cluster where the proteolytic pseudomonads resided in.

The first cluster consisted of milk isolates 0-0, 8-1, 10-6, 2-1, 2-2, 2-4, 6-0, B16, 10-1, and the reference strains *P. fluorescens* (ATCC 948) and *P. fluorescens* biotype A (ATCC 17397). Also in this group were the species *P. fluorescens* biotype A, *P. aureofaciens*, *P. fluorescens* biotype C, *P. fluorescens* biotype B and *P. chlororaphis* as described in the Bergey's Manual of Determinative Bacteriology (1974). These species formed a cluster at the 82% S_{SM}. The milk isolates 4-1, 6-3, A14, 10-7, 8-2, 8-4, and 10-0 were in another cluster along with the reference strain *P. fragi* (ATCC 4976). This cluster was distinct and did not join the other clusters until the 67% similarity level (% S_{SM}).

All isolates belonging to the first cluster were fluorescent *Pseudomonas* species. *P. chlororaphis* and *P. aureofaciens* were regarded as *P. fluorescens* biotypes D and E respectively by Stanier et al. (1966). Besides producing

Fig. 8. Dendrogram showing relationship of proteolytic pseudomonads from raw milk. Strains with asteriks are the strains as described in the Bergey's Manual of Determinative Bacteriology (1974).


*P. aeruginosa
*P. putida
*P. stutzeri
*P. mendocina
P. putida
*P. alcaligenes
*P. pseudoalcaligenes
*P. testosteroni
*P. acidovorans
*P. ruhlandi
*P. palleronii
*P. lemoignei
*P. solanacearum
*P. fluorescens biotype A
*P. aureofaciens
0-0
8-1
10-6
2-1
2-2
2-4
6-0
B16
P. fluorescens biotype A
*P. fluorescens biotype C
P. fluorescens
10-1
*P. fluorescens biotype B
*P. chlororaphis
C10
*P. pseudomallei
*P. caryophylli
*P. cepacia
*P. marginata
*P. mallei
*P. syringae
*P. cichorii
*P. delafieldii
*P. facilis
*P. saccharophilia
*P. flava
4-1
6-3
A14
P. fragi
10-7
8-4
8-2
10-0
0
20
40
60
80
100
% SIMILARITY

fluorescent pigment, P. chlororaphis produces a green insoluble pigment (chlororaphin) whereas P. aureofaciens produces an orange diffusible pigment (phenazine-1-carboxylic acid). These two pigments were absent from all of the milk isolates on both medium A and B of King et al. (1954).

All the milk isolates, residing in the first cluster, shared the ability to use 25 of the 38 compounds tested as the sole sources of carbon and nitrogen. They could not utilize maltose, lactose, butyrate, propyleneglycol, phenol as the sole source of carbon, and nicotinate and pantothenate as the sole source of nitrogen.

Every member of the first cluster, including strains described as in the Bergey's Manual of Determinative Bacteriology (1974), could use the following compounds as the sole source of carbon: ribose, glucose, mannose, trehalose, gluconic acid, acetate, propionate, succinate, malate, lactate, citrate, pyruvate, mannitol, glycerol, alanine, valine and arginine. They could not use maltose, lactose, phenol as the sole source of carbon, and nicotinate as the sole source of nitrogen.

All milk isolates could use galactose as a carbon source whereas the species described as P. fluorescens biotype A and P. chlororaphis, by Stanier et al. (1966), could not. However P. fluorescens biotypes B, C, and P. aureofaciens could utilize galactose. The ability to denitrify was demonstrated by P. fluorescens biotypes B, C, and P. chlororaphis, but not by P. fluorescens biotype A and P. aureofaciens (Stanier et al.,

1966). This property was not demonstrated by all of the milk isolates. It is not surprising that the milk isolates had a few nutritional properties different from those described by Stanier et al. (1966) or in the Bergey's Manual of Determinative Bacteriology (1974). Juffs (1973) reported that 103 of the 121 isolates from raw milk were unable to denitrify. Although utilization of trehalose was considered by Stanier et al. (1966) to be an important characteristic of P. fluorescens, nine percent of the isolates Juffs (1973) examined were unable to utilize trehalose.

The internal subdivision of P. fluorescens is far from satisfactory. The subdivision in biotypes is primarily based on two salient properties namely the synthesis of levan from sucrose and capacity for denitrification. Some characters correlate fairly well with these two, but the delimitation of the biotypes in some cases is rather unclear (Palleroni, 1975). The strains of biotype A are capable of levan synthesis but not of denitrification. The strains assigned to biotype B are phenotypically more heterogenous than those of biotype A, and are capable of both levan formation and denitrification. The strains incapable of levan production but capable of denitrification are assigned to biotype C. The biotype is heterogenous and can be divided into several subgroups (Palleroni and Doudoroff, 1972). The group of miscellaneous strains assigned to biotype G is heterogenous in nutritional properties. They are also incapable of denitrification and levan synthesis. Strains of other biotypes which have lost, by

mutation, one or both of the basic properties of characterization would be placed in this biotype. The species P. chlororaphis was originally described as P. fluorescens biotype D by Stanier et al. (1966). Strains of this species are capable of levan formation and denitrification. The species P. aureofaciens was originally assigned as P. fluorescens biotype E (Stanier et al., 1966). Strains of this species are capable of levan production but incapable of denitrification. The reason why P. chlororaphis and P. aureofaciens were granted species status was because they produced very characteristic phenazine pigments (Stanier et al., 1966). Nucleic acid homology studies showed that these two species shared a high level of DNA homology (Palleroni and Doudoroff, 1972). Liu (1960), in a serological study on the extracellular antigens of Pseudomonas strains, found that P. aureofaciens and P. chlororaphis showed extensive cross-reactions, which suggested a close relationship among these species. P. fluorescens is probably the most complex species of the genus Pseudomonas. Most extensive studies done on P. fluorescens were of soil, plant or water origin. Information on P. fluorescens in milk is still lacking. The classification of the Pseudomonas species presented in this thesis is still incomplete. A study of more characters and on additional strains would perhaps result in a more precise classificaion of isolates and the creation of new subgroups. Above all, data from nucleic acid homology studies will provide more valuable information to the current characterization presented here.

All the milk isolates residing in the cluster containing the reference *P. fragi* (ATCC 4976) did not produce any fluorescent pigment. They were all able to utilize xylose, malate, lactate, citrate, pyruvate, alanine, serine, valine and arginine as a sole carbon source. They could not utilize pantothenate as the sole nitrogen source. The nutritional spectrum of this cluster was not as broad as the *P. fluorescens* cluster. They could not utilize rhamnose, maltose, lactose, propionate, butyrate, inositol, adonitol, propyleneglycol, methanol and ethanol. Of all the strains in this cluster, only one could denitrify. The *P. fragi* related milk isolates were all able to breakdown milk caseins at 4 C and hydrolyze butterfat. Colonies formed on skim milk agar by members of this cluster were more slimy in appearance than those of *P. fluorescens* species.

P. fragi is not assigned as a *Pseudomonas* species in the Bergey's Manual of Determinative Bacteriology (1974). It is listed under addendum I to the genus. In the seventh edition of the Bergey's Manual (Breed et al., 1957) *P. fragi* was described as possessing the following characteristics: no production of soluble pigments, liquefaction of gelatin, growth at 25 C but not at 37 C, absence of the nitrate reduction property, and acid production from glucose, galactose and arabinose. The sources of isolation were described as from milk and other dairy products, dairy utensils and water.

This study showed that half of the proteolytic milk pseudomonads belonged to the *P. fluorescens* group and the other

half to the P. fragi group. Muir et al. (1979) reported that on average 43% of milk pseudomonads were of the fluorescent type. Characterization of these fluorescent strains were not done. Juffs (1973) reported that 72% of the pseudomonads in raw milk were P. fluorescens. Samagh and Cunningham (1972) reported that of the 182 pseudomonads studied, 98 strains were identified as P. fluorescens and P. aeruginosa-like organisms. Isolation of P. fragi-like organisms from milk was only reported by a few researchers. Law et al. (1976) reported that the highest incidence of lipolytic activity among the psychrotrophic Gram-negative flora of commercial raw milk was found in strains of P. fluorescens and P. fragi. Samagh and Cunningham (1972) suspected that 41 non-fluorescent cultures (out of 182 pseudomonads) were related to P. fragi and P. putrefaciens. They did not include these strains as references in their study. Isolation of P. fragi from milk was also reported by Overcast (1968).

The finding of the distinct cluster consisting of the milk isolates 4-1, 6-3, A14, 10-7, 8-4, 8-2, 10-0, and the reference strain P. fragi (ATCC 4976) is quite interesting. Taxonomic study of P. fragi-like organisms from milk has not been published. P. fragi is the major pseudomonad responsible for meat spoilage. Shaw and Latty (1982) performed a numerical taxonomic study of Pseudomonas strains from spoiled meat. The Pseudomonas strains were shown to reside in four clusters. Non-fluorescent strains were contained in two closely related clusters which were identified with P. fragi (NCIB 8542). The

other two clusters were distinct from P. fluorescens (NCIB 9046), P. putida (NCIB 9494) and other reference strains examined. All members of the four clusters produced acid oxidatively, grew at 4 C but not at 41 C, did not accumulate poly-β-hydroxybutyrate, did not denitrify and did not produce levan from sucrose. It is quite surprising that the reference P. fluorescens strain (NCIB 9046, biotype A) that they examined did not produce levan from sucrose. The P. fragi-like strains were all non-fluorescent, able to hydrolyze gelatin (20 C) but did not show phospholipase activity. The two clusters, consisting of P. fragi-like strains, were distinguished from one another by their pattern of carbon source utilization. Most of the members in the cluster, where the reference strain P. fragi (NCIB 8542) resided in, could hydrolyze casein but could not utilize galactose as sole carbon source. Members of the other cluster, regarded as a biotype of P. fragi, were able to utilize galactose but few could hydrolyze casein. Only three strains, from the two clusters, could hydrolyze Tween 80 (Shaw and Latty, 1982).

Results presented in the present study confirm the heterogenous nutritional properties of the P. fragi group. Only one strain in this study could denitrify and few could use galactose as sole carbon source.

Lysenko (1961) attempted to characterize 126 strains representing 46 different Pseudomonas species. The strains were isolated mainly from humans, animals, plants, soil and water. Lysenko's (1961) results indicated that P. fragi was distinct

from P. fluorescens, P. putida, and P. aeruginosa. The species P. fragi showed the following characteristics: growth at 5 C but not at 42 C, absence of fluorescent pigments, inability to hydrolyze casein and gelatin at 28 C, ability to hydrolyze olive oil, lack of denitrification property, utilization of galactose and citrate as carbon sources, failure to use glucose, lactose, maltose and lactate as sole carbon source.

An identification key to the species P. fragi is not given in the eigth edition of the Bergey's Manual of Determinative Bacteriology (1974) nor in the diagnostic tables of Cowan and Steel (1965). Characterization of P. fragi-like strain from raw milk would be impossible unless identification keys other than Bergey's Manual (1974) or Cowan and Steel (1965) are used. Results presented here warrant future research on the taxonomic status of P. fragi, especially from food sources.

Table 2 shows some hydrolytic characteristics of the milk isolates. The milk isolates identified with P. fluorescens were all capable of hydrolyzing milk proteins at 4 C and 22 C. This property was demonstrated at 32 C by all the milk isolates except strain 10-1. Gelatinase activity at 22 C was shown by all but one of the isolates. However hydrolysis of gelatin at 4 C was only exhibited by strains 10-6, 2-1, 2-4, 6-0, and B16. All the isolates also showed lipase and phospholipase activities at 22 C.

The isolates identified with P. fragi were somewhat different in character. Proteolysis was detected with all isolates at 4 C but not at 32 C. The majority of these isolates

Table 2. Hydrolytic characteristics of the milk pseudomonads

CHARACTERISTICS	STRAINS																				
	0 - 0	8 - 1	10 - 6	2 - 1	2 - 2	2 - 4	6 - 0	B16	P. FLUORESCENS BIOTYPE A	10 - 1	P. FLUORESCENS	C10	4 - 1	6 - 3	A14	10 - 7	8 - 4	8 - 2	10 - 0	P. FRAGI
MILK PROTEINS HYDROLYSIS (4°C)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MILK PROTEINS HYDROLYSIS (22°C)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+
MILK PROTEINS HYDROLYSIS (32°C)	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
GELATIN HYDROLYSIS (4°C)	-	-	+	+	-	+	+	+	-	-	-	-	-	+	-	-	+	+	-	-
GELATIN HYDROLYSIS (22°C)	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-
BUTTERFAT HYDROLYSIS (22°C)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TWEEN 80 HYDROLYSIS (22°C)	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-	+	-	+	-
LECITHIN HYDROLYSIS (22°C)	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-

showed proteinase activity at 22 C. Gelatinase activity was detected with three of the isolates at 4 C but none were gelatinase positive at 22 C. These P. fragi-like isolates showed lipase activity but no phospholipase activity.

From table 2, we can see that the nature of the substrate and the conditions under which the test is being conducted are critical. If proteinase activity was tested at 32 C, all the P. fragi-like isolates would be negative. P. fragi-like strains would be regarded as non-proteolytic, if gelatin was used instead of milk proteins as the substrate with an incubation temperature of 22 C. The P. fragi related milk isolates were all able to hydrolze butterfat but not Tween 80. Numerous studies on the lipase activity of P. fragi have been reported (Nashif and Nelson, 1953; Law et al., 1976; Griffiths et al., 1981). However only strains A14, 8-4, and 10-0 were able to hydrolyze Tween 80. This agrees with Shaw and Latty (1982) that few of the P. fragi-like strains were able to hydrolyze Tween 80. It is apparent that butterfat hydrolysis is a better test for the presence of lipase than hydrolysis of Tween 80.

The characteristics discussed and tabulated in this study are for collection of information on the general properties of these milk isolates. More work on a larger collection of isolates will be necessary before a key for the differentiation of the clusters of milk isolates can be determined. It should be remembered that the structure of a determinative key may change when new species or species from other origins are included.

Richardson (1981) classified the milk isolate as P. fluorescens B52 because it could utilize trehalose and β-alanine as sole carbon source, and it could grow at 4 C but not at 41 C. All P. fluorescens related isolates in this study could use trehalose as the sole carbon source. Juffs (1973) reported that 91% of the P. fluorescens related strains could utilize trehalose. Other studies indicated that 67% (Shaw and Latty, 1982) and 52% (Klinge, 1960) of the strains could utilize trehalose as a carbon source. Strains lacking the ability to grow on trehalose will not be assigned to the P. fluorescens group.

According to Stanier et al. (1966) the species P. aeruginosa is easily distinguished from other fluorescent pseudomonads. The characters most commonly used for its recognition are: pyocyanine production; growth at 41 C but not at 4 C; and its narrower nutritional spectrum as compared to P. fluorescens. P. aeruginosa strains are remarkably uniform in phenotypic properties and in nucleic acid homology (Stanier et al., 1966). Many phages are specific for the strains of this species. However, Samagh and Cunningham (1972) reported an aeruginosa-like group which was related to P. fluorescens. They concluded that members of this group were psychrotrophic aeruginosa-like organisms that lost the ability to grow at 41 C and to produce pyocyanine. Future research is needed to confirm the presence of P. aeruginosa-like strains.

P. putida, as described by various authors, is not able to demonstrate proteinase and lipase activities (Rhodes, 1959;

Lysenko, 1961; Stanier et al., 1966). However studies on the proteinase activity of P. putida strains isolated from raw milk have been published (Law et al., 1979 a, b). P. putida related strains isolated from raw milk were reported to be non-proteolytic (Juffs, 1973). Shaw and Latty (1982) observed the same property from their strains isolated from meat. Whether a false identification had been made or a mutant of the species P. putida was isolated from milk remains to be seen. Nevertheless care must be taken when an identification key is needed for rapid characterization.

B. Growth of proteolytic pseudomonads in sterile milk

Eight proteolytic isolates, from raw milk, identified to be Pseudomonas species were grown in 10% RSM at 4 C. Proteinase activities were detected in 10% RSM inoculated with strains 0-0, 2-1, 8-1, and 8-2 when the bacterial populations reached 10^8 - 10^9 cfu/ml (Fig. 9 a, c, d and Fig. 10c). No proteinase activity was detected from strains 4-1, 6-3, and 10-0 even though the proteolytic populations reached 10^9 cfu/ml (Fig. 10 a, b, d). A slight increase in proteinase activity was detected from strain 2-2 after 19 d at 4 C (Fig. 9b). It is evident that bacterial count alone cannot indicate when proteolysis will occur. Reports on populations needed to detect proteolysis varied, ranging from 10^5 - 10^9 cfu/ml. No relationship between the level of bacterial population and proteolytic activity was also observed by Elliot et al. (1974), Law (1979), Cousin (1982)

Fig. 9. Growth and proteinase activity (▲) in 10% RSM inoculated with (a) Isolate 0-0; (b) Isolate 2-1; (c) Isolate 2-2; (d) Isolate 8-1.
Total psychrotrophs (■) ; proteolytic psychrotrophs (□).
Proteinase activity is defined as the increase in the concentration of TCA-soluble free amino groups per milliliter of milk after incubation at 40 C for 2 h.

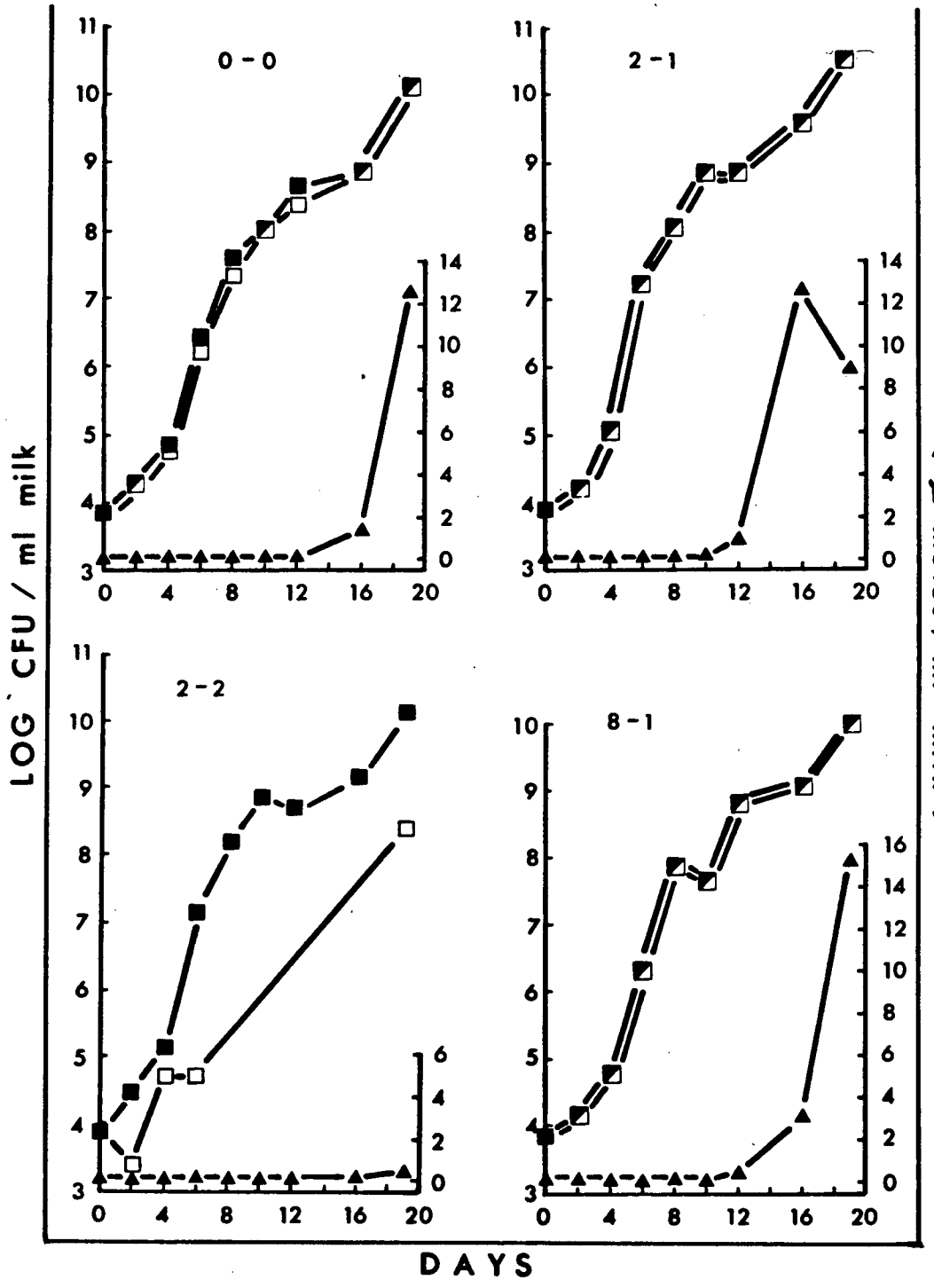
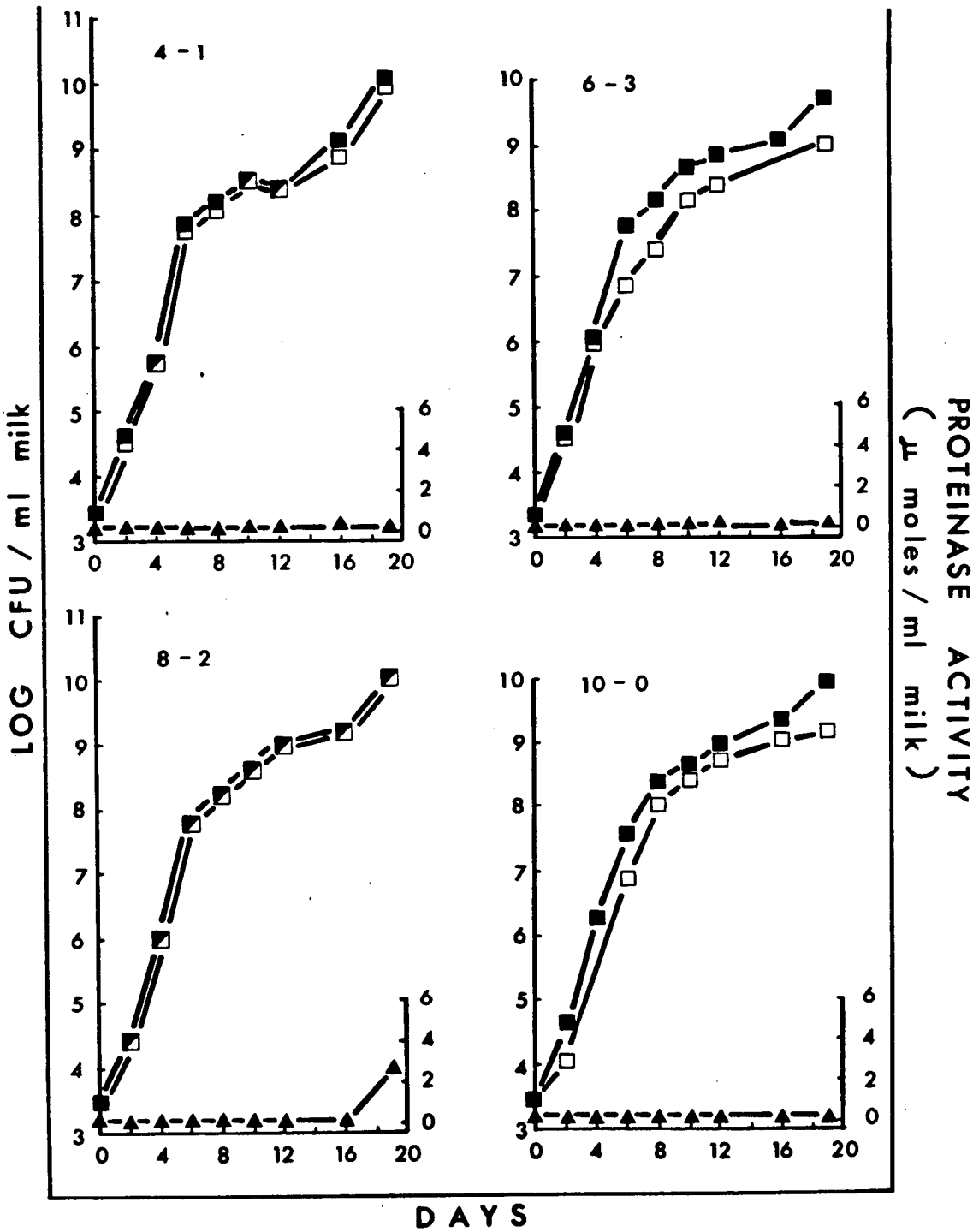

0 - 0
2 - 1
2 - 2
8 - 1
LOG CFU / ml milk
PROTEINASE ACTIVITY
(μ moles / ml milk)
DAYS

Fig. 10. Growth and proteinase activity (▲) in 10% RSM inoculated with (a) Isolate 4-1; (b) Isolate 6-3; (c) Isolate 8-2; (d) Isolate 10-0.
Total psychrotrophs (■) ; proteolytic psychrotrophs (□). Proteinase activity is defined as the increase in the concentration of TCA-soluble free amino groups per milliliter of milk after incubation at 40 C for 2 h.


4 - 1
6 - 3
8 - 2
10 - 0
LOG CFU / ml milk
PROTEINASE ACTIVITY
(μ moles / ml milk)
DAYS
0
4
8
12
16
20
3
4
5
6
7
8
9
10
11
0
2
4
6

and Cliffe and Law (1982).

Proteolysis was not detected from strains 4-1, 6-3, and 10-0 because they either had not produced sufficient proteinases that could be detected by the fluorescamine method or they had not started to produce proteinases. It has been shown that the keeping quality of milk is dependent on the number of bacteria present in the milk, length of the lag phase of growth, rate of growth at storage temperature and type of bacteria present (Smith et al., 1972). A longer storage time at 4 C is needed before a marked increase in proteinase activity could be detected from strain 2-2.

Electrophoregrams showed that β-casein was more susceptible to proteolysis caused by strains 0-0, 2-1, 8-1, and 8-2 (Fig. 11 and 12). Extensive breakdown of β- and α_s-caseins were demonstrated by strains 0-0, 2-1, and 8-1 (Fig. 11 and 12), which also showed the largest increase in proteinase activity (Fig. 9). Strains 2-2, 4-1, 6-3, and 10-0 did not cause any noticeable changes on the α_s- and β-caseins (Fig. 13). This is the reason why no proteinase activities, using the fluorescamine method, were detected from these strains.

Milk protein degradation by proteolytic psychrotrophs has been extensively reviewed (Law, 1979; Cousin, 1982). Gebre-Egziabher et al. (1980 b) reported that κ- and β-caseins were more susceptible to proteolysis by Pseudomonas species while the α_s-casein was less affected. At the end of 15 days at 7 C, electrophoresis showed no remaining traces of κ- and β-caseins while a considerable loss of α_s-casein. Law et al. (1977)

Fig. 11. Electrophoretic profiles of milk proteins caused by isolates 0-0 and 2-1 after 0, 6, 16 and 19 days of storage in 10% RSM at 4 C.

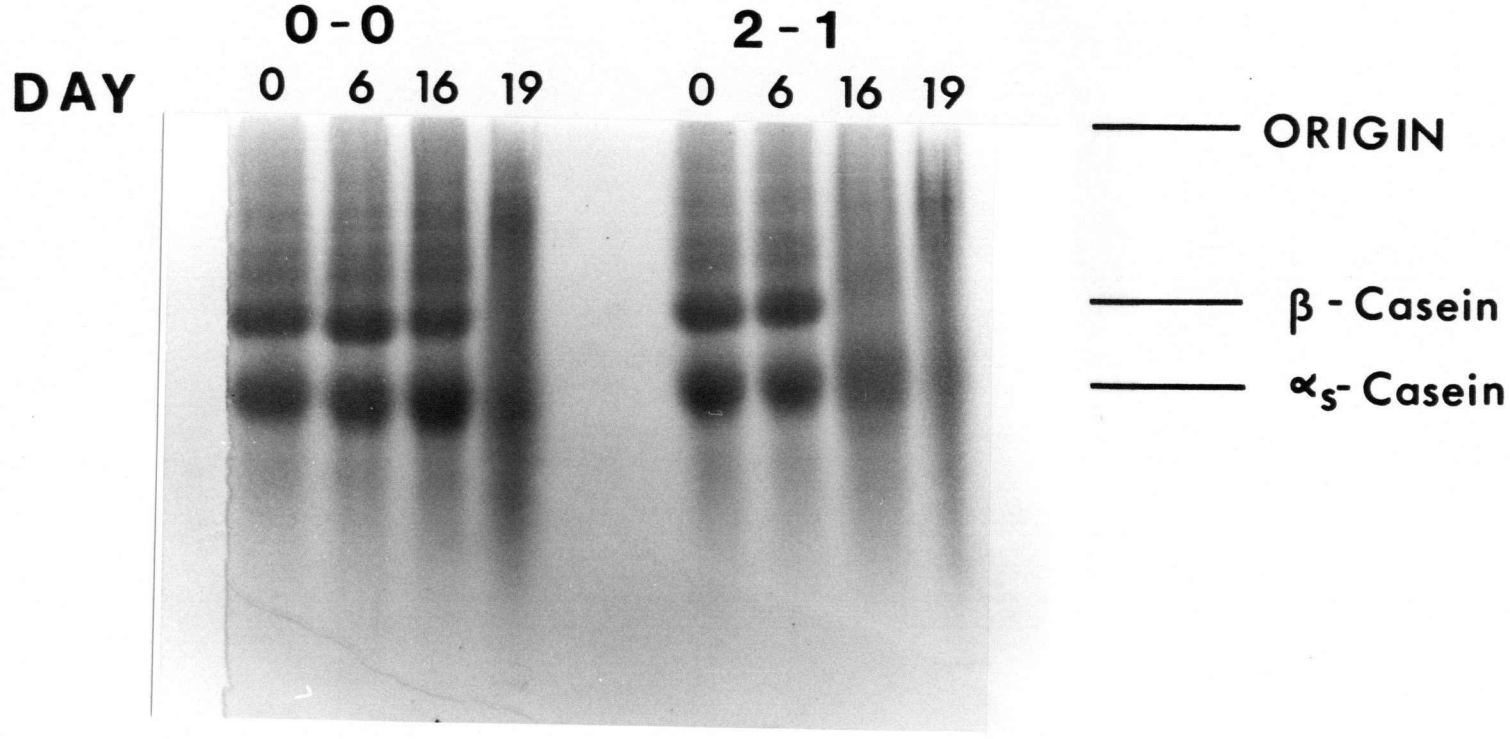

0-0
2-1
DAY
0 6 16 19
0 6 16 19
ORIGIN
β - Casein
α_s - Casein

Fig. 12. Electrophoretic profiles of milk proteins caused by isolates 8-1 and 8-2 after 0, 6, 16 and 19 days of storage in 10% RSM at 4 C.

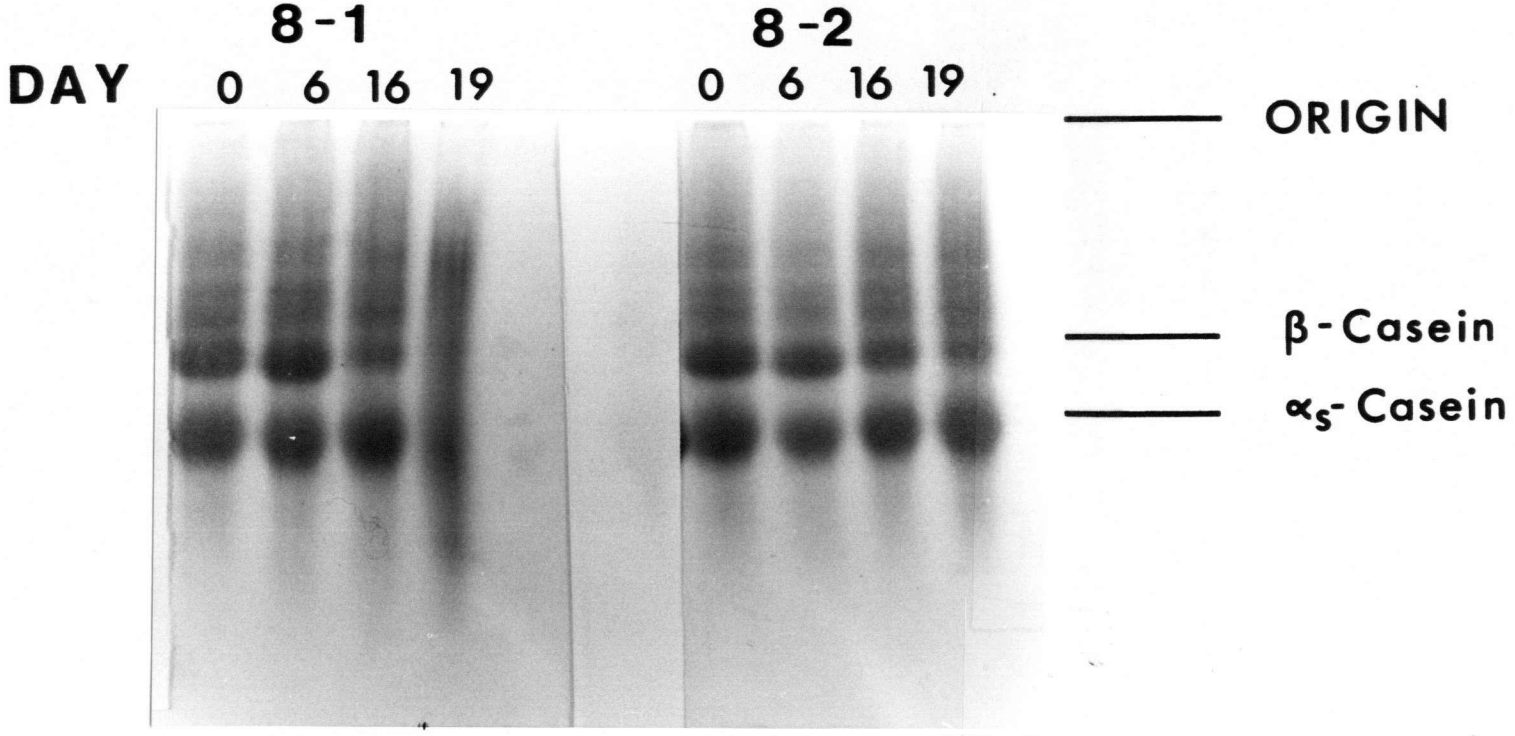
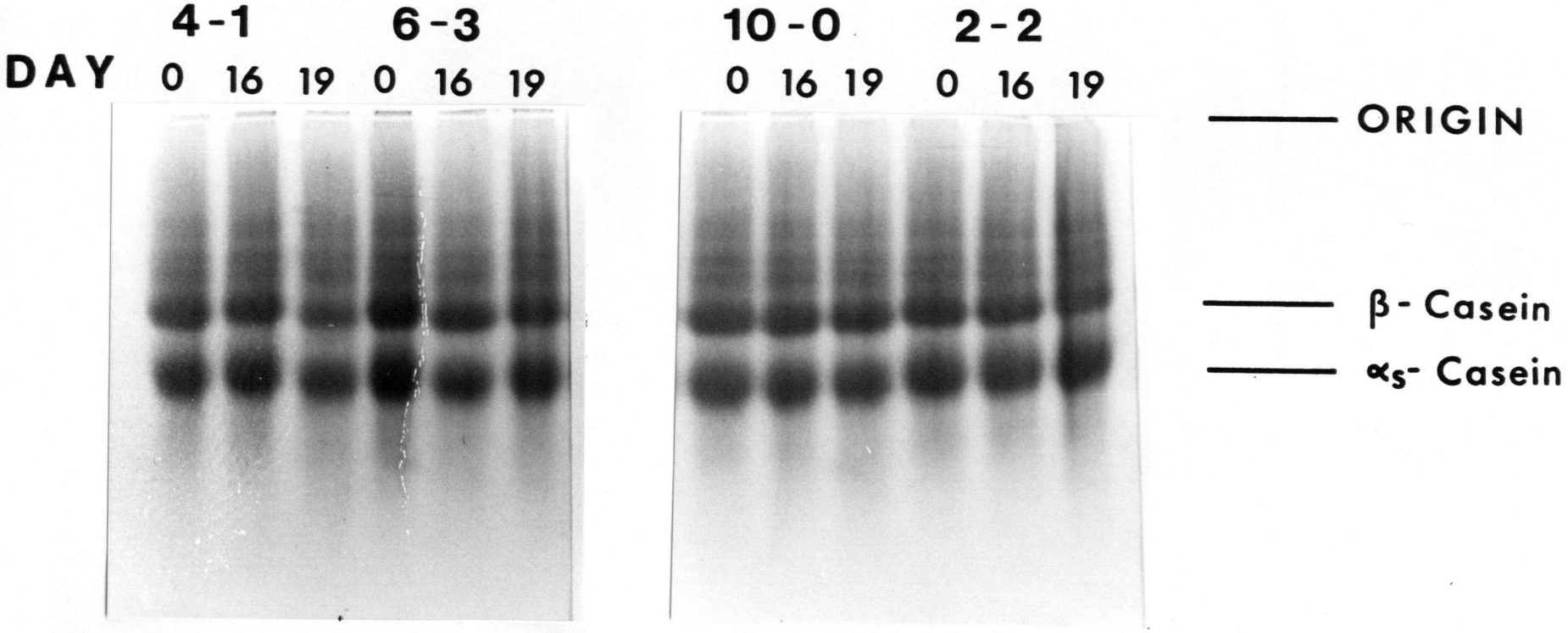

8-1
8-2
DAY
0 6 16 19
0 6 16 19
ORIGIN
β-Casein
α_s-Casein

Fig. 13. Electrophoretic profiles of milk proteins caused by isolates 4-1; 6-3; 10-0 and 2-2 after 0, 16 and 19 days of storage in 10% RSM at 4 C.


4-1
6-3
10-0
2-2
DAY
0 16 19 0 16 19
0 16 19 0 16 19
ORIGIN
β- Casein
αs- Casein

studied gelation of ultra-high-temperature-sterilized milk by proteases from P. fluorescens, and reported that β- and κ-caseins were extensively degraded in gelled milk samples and some loss of a_s-casein was seen. Cousin and Marth (1977) noted that Pseudomonas species hydrolyzed both a_s- and β-caseins while κ-casein was not affected. Electrophoresis showed both a_s- and β-caseins disappeared after 9 days at 7 C. DeBeukelar et al. (1977) observed that a_s- and β-caseins were degraded in skim milk inoculated with Pseudomonas species. In their study on the changes of milk protein by Pseudomonas species, Yanagiya et al. (1973) also found that a_s-casein disappeared after 14 days at 5 C.

CONCLUSIONS

In conclusion, the proteolytic pseudomonads in milk could be classified into two clusters. The first cluster contained *P. fluorescens* (at 82% S_{SM}) while the second contained *P. fragi* (at 80% S_{SM}). The isolates identified with *P. fluorescens* could: grow at 4 C but not at 41 C; hydrolyze milk proteins, butterfat; could produce phospholipase. They produced diffusible pigment but failed to denitrify. They could not use lactose as sole carbon source but could use galactose. The cluster containing *P. fragi* could: grow at 4 C but not at 41 C; hydrolyze milk proteins and butterfat but possessed no phospholipase activity. No fluorescent pigment was produced and only one strain could denitrify. No members in this cluster could utilize lactose as sole carbon source but few could use galactose.

No relationship was found between proteolytic psychrotrophs populations and proteolysis in milk stored at 4 C. Proteinase activities were detected, when proteolytic psychrotrophic counts reached 10^8 cfu/ml, from strains 0-0, 2-1, 8-1, and 8-2 but not from strains 2-2, 4-1, 6-3, and 10-0.

REFERENCES

Adams, D.M., Barach, J.T., and Speck, M.L. 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci. 58:828.

Audrey, J., Jr. and Frazier, W.C. 1959. Psychrophiles in milk held two days in farm milk tanks. J. Dairy Sci. 42:1781.

Barritt, M. 1936. The intensification of the Voges - Proskauer method by the addition of alpha-naphthol. J. Path. Bacteriol. 42:441.

Beech, F.W., Carr, J.G., and Codner, R.C. 1955. A multipoint inoculator for plating bacteria or yeasts. J. Gen. Microbiol. 13:408.

Bergey's Manual of Determinative Bacteriology. 1974. 8th ed. Buchanan, E.R. and Gibbons, N.E. eds. Williams and Wilkins Co., Baltimore, MD. pp 217-243.

Breed, R.S., Murray, E.G.D., and Smith, N.R. 1957. In Bergey's Manual of Determinative Bacteriology. 7th ed. Williams and Wilkins Co., Baltimore, MD. pp 90-152.

Casas, I.A., Leon, N., and Izquierdo, P. 1977. Microtiter technique for enumeration of mesophiles, psychrotrophs and coliforms in raw and pasteurized milk. J. Food Prot. 40:795.

Castell, J.V., Cervera, M., and Marco, R. 1979. A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A reexamination of the conditions of reaction. Anal. Biochem. 99:379.

Chrisope, G.L., Fox, C.W., and Marshall, R.T. 1976. Lecithin agar for detection of microbial phospholipases. Appl. Environ. Microbiol. 31:784.

Clark, W.M. and Lubs, H.A. 1915. The differentiation of bacteria of the colon - aerogenes family by the use of indicators. J. Infect. Dis. 17:160.

Cliffe, A.J. and Law, B.A. 1982. A new method for the detection of microbial proteolytic enzymes in milk. J. Dairy Res. 49:209.

Cousin, M.A. 1982. Presence and activity of psychrotrophic microorganism in milk and dairy products: A review. J. Food Prot. 45:172.

Cousin, M.A. and Marth, E.H. 1977. Changes in milk proteins caused by psychrotrophic bacteria. Milchwissenschaft 32:337.

Cowan, S.T. and Steel, K.J. 1965. In Manual for the Identification of Medical Bacteria. 1st ed. Cambridge University Press, England. pp 61-82.

Cowan, S.T. and Steel, K.J. 1974. In Manual for the Identification of Medical Bacteria. 2nd ed. Cambridge University Press, England. pp 161-180.

DeBeukelar, N.J., Cousin, M.A., Bradley, R.L., Jr., and Marth, E.H. 1977. Modification of milk proteins by psychrotrophic bacteria. J. Dairy Sci. 60:857.

Dowson, W.J. 1949. In Manual of Bacterial Plant Diseases. 1st ed. Black, A. and Black, C. London, England. p 36.

Elliott, J.A., Emmons, D.B., and Yates, A.R. 1974. The influence of bacterial quality of milk on the properties of dairy products. A review. Can. Inst. Food Sci. Technol. J. 7:32.

Fox, C.W., Chrisope, G.L., and Marshall, R.T. 1976. Incidence and identification of phospholipase C - producing bacteria in fresh and spoiled homogenized milk. J. Dairy Sci. 59:1857.

Fung, D.Y.C. and Hartman, P.A. 1972. Rapid characterization of bacteria, with emphasis on Staphylococcus aureus . Can. J. Microbiol. 18:1623.

Gebre-Egziabher, A., Humbert, E.S., and Blankenagel, G. 1980a. Heat stable proteases from psychrotrophs in milk. J. Food Prot. 43:197.

Gebre-Egziabher, A., Humbert, E.S., and Blankenagel, G. 1980b.

Hydrolysis of milk proteins by microbial enzymes. J. Food Prot. 43:709.

Griffiths, M.W., Phillips, J.D., and Muir, D.D. 1981. Thermostability of proteases and lipases from a number of species of psychrotrophic bacteria of dairy origin. J. Appl. Bacteriol. 50:289.

Hugh, R. 1973. In Manual of Clinical Microbiology. 2nd ed. American Society for Microbiology, Bethesda, MD. pp 175-190.

Hugh, R. and Leifson, E. 1953. The taxonomic significance of fermentive versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. J. Bacteriol. 66:24.

Jones, A. and Richards, T. 1952. Night blue and Victoria blue as indicators in lipolysis media. Proc. Soc. Appl. Bacteriol. 15:82.

Juffs, J.S. 1973. Identification of Pseudomonas species isolated from milk produced in south eastern Queensland. J. Appl. Bacteriol. 36:585.

Juffs, J.S. 1974. Influence of proteinases produced by Pseudomonas aeruginosa and Pseudomoas fluorescens on manufacture and quality of Cheddar cheese. Aust. J. Dairy Technol. 29:74.

Kaneko, T., Holder-Franklin, M., and Franklin, M. 1977. Multiple syringe inoculator for agar plates. Appl. Environ. Microbiol. 33:982.

King, E.O., Ward, M.K., and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301.

Klinge, K. 1960. Differential techniques and methods of isolation of Pseudomonas . J. Appl. Bacteriol. 23:442.

Kohn, J. 1953. A preliminary report of a new gelatin liquefaction method. J. Clin. Microbiol. 6:249.

Kovacs, N. 1956. Identification of Pseudomonas pyocyanea by

the oxidase reaction. Nature, London 178:703.

Law, B.A. 1979. Reviews of the progress of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. 46:573.

Law, B.A., Sharpe, M.E., and Chapman, H.R. 1976. The effect of lipolytic Gram-negative psychrotrophs in stored milk on the development of rancidity in Cheddar cheese. J. Dairy Res. 43:259.

Law, B.A., Andrews, A.T., and Sharpe, M.E. 1977. Gelation of ultra-high-temperature-sterilized milk by proteases from a strain of Pseudomonas fluorescens isolated from raw milk. J. Dairy Res. 44:145.

Law, B.A., Andrews, A.T., Cliffe, A.J., Sharpe, M.E., and Chapman, H.R. 1979a. Effect of proteolytic raw milk psychrotrophs on Cheddar cheese-making with stored milk. J. Dairy Res. 46:497.

Law, B.A., Cousins, C.M., Sharpe, M.E., and Davies, F.L. 1979b. In Cold Tolerant Microbes in Spoilage and the Environment. Russell, A.D. and Fuller, R. eds. Academic Press, New York. pp 137-152.

Lee, J.S. 1976. In Compendium of Methods for the Microbiological Examination of Foods. Speck, M.L. ed. A.P.H. Inc., Washington, DC. pp 180-183.

Liu, P.V. 1960. Identification of pathogenic pseudomonads by extracellular antigens. J. Bacteriol. 81:28.

Lysenko, O. 1961. Pseudomonas - An attempt at a general classification. J. Gen. Microbiol. 25:379.

McKellar, R.C. 1981. Development of off-flavors in ultra-high temperature and pasteurized milk as a function of proteolysis. J. Dairy Sci. 64:2138.

Muir, D.D., Phillips, J.D., and Dalgleish, D.G. 1979. The lipolytic and proteolytic activity of bacteria isolated from raw milk. J. Soc. Dairy Technol. 32:19.

Nakai, S., Wilson, H.K., and Herreid, E.O. 1964. Assaying sterile concentrated milk for native proteolytic enzymes. J. Dairy Sci 47:754.

Nashif, S.A. and Nelson, F.E. 1953. The lipase of Pseudomonas fragi . I. Characterization of the enzyme. J. Dairy Sci. 36:459.

Niven, C.F., Jr., Smiley, K.L., and Sherman, J.M. 1942. The hydrolysis of arginine by streptcocci. J. Bacteriol. 43:651.

Numerical Taxonomy System of Multivariate Statistical (NT-SYS) Programs. 1980. Rohlf, F.J., Kishpaugh, J., and Kirk, D. Department of Ecology and Evolution. The State University of New York at Stony Brook, NY.

Otte, I., Tolle, A., and Hahn, G. 1979. Microbial analysis of milk and milk products. 2. Miniaturized primary tests for identification of genera. Milchwissenschaft 34:152.

Otto, L.A. and Blachman, U. 1979. Nonfermentative bacilli: Evaluation of three systems for identification. J. Clin. Microbiol. 10:147.

Overcast, W.W. 1968. Psychrotrophic microorganisms and keeping quality of milk and its products. J. Dairy Sci. 51:1336.

Palleroni, N.J. 1975. In Genetics and Biochemistry of Pseudomonas . Clarke, P.H. and Richmond, M.H. eds. John Wiley and Sons Ltd., London, England, pp 1-36.

Palleroni, N.J. and Doudoroff, M. 1972. Some properties and taxonomic subdivisions of the genus Pseudomonas . An. Rev. Phytopathol. 10:73.

Rhodes, M.E. 1959. The characterization of Pseudomonas fluorescens . J. Gen. Microbiol. 21:221.

Richardson, B.C. 1981. The purification and characterization of a heat-stable protease from Pseudomonas fluorescens B52. N. Z. J. Dairy Sci. Technol. 16:195.

Richardson, B.C. and Te Whaiti, I.E. 1978. Partial characterization of heat-stable extracellular proteases of some psychrotrophic bacteria from raw milk. N. Z. J. Dairy Sci. Technol. 13:172.

Richardson, B.C. and Newstead, D.F. 1979. Effect of heat-stable proteases on the storage life of UHT milk. N. Z. J. Dairy Sci. Technol. 14:273.

Samagh, B.S. and Cunningham, J.D. 1972. Numerical taxonomy of the genus Pseudomonas from milk and milk products. J. Dairy Sci. 55:19.

Shaw, B.G. and Latty, J.B. 1982. A numerical taxonomic study of Pseudomonas strains from spoiled meat. J. Appl. Bacteriol. 52:219.

Smith, D.A. 1961. A multiple inoculation device for use with fluids. J. Appl. Bacteriol. 24:131.

Smith, K.L., Mull, L.E., Lane, C.B., and Baggott, A.J., Jr. . 1972. Keeping quality of milk exposed to high temperature as experienced during transport in automobiles. J. Milk Food Technol. 35:588.

Sneath, P.H.A. and Sokal, R.R. 1973. In Numerical Taxonomy. W.H. Freeman and Co., San Francisco, CA. pp 230-234.

Stadhouders, J. 1975. Microbes in milk and dairy products. An ecological approach. Neth. Milk Dairy J. 29:104.

Stanier, R.Y., Palleroni, N.J., and Doudoroff, M. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159.

Tayfour, A., Milliere, J.B., and Veillet-Poncet, L. 1982. Growth and proteolytic activity of Pseudomonas fluorescens 28P12 on milk retentates stored at low temperature. Milchwissenschaft 37:720.

Tompkin, R.B. 1973. Refrigeration temperature as an environmental factor influencing the microbial quality of food. A review. Food Technol. 27:54.

Thomas, S.B. and Thomas, B.F. 1973. Psychrotrophic bacteria in refrigerated bulk-collected raw milk. Dairy Ind. 38:61.

Witter, L.D. 1961. Psychrophilic bacteria. A review. J. Dairy Sci. 44:983.

Yanagiya, T., Mikami, M., and Miura, H. 1973. Changes of milk protein by psychrotrophic organisms. J. Agr. Chem. Soc. Jap. 47:259.