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Determination of proteolytic activity in different milk systems

S. Bendicho, G. Martí, T. Hernández, O. Martín*

CeRTA-UTPV, Departamento de Tecnología de Alimentos, Universidad de Lleida, Avda. Alcalde Rovira Roure 177, 25198 Lleida, Spain

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Abstract

A method for the determination of proteolytic activity in whole milk, skim milk or in a solution similar to skim milk ultrafiltrate was optimized and validated. An artificial substrate (azocasein) was used to quantify the enzyme activity through the release of a chromogenic product that was measured spectrophotometrically after clarifying the samples by the addition of trichloracetic acid. The method gave linear results in the range of 0–50 mU/ml of added *Bacillus subtilis* protease and the least detection and quantification limits were 2.29 and 7.64 mU/ml of protease, respectively. The precision, measured as relative standard deviation, was between 1.14 and 7.99% and mean recovery ranged between 99 and 104%. Reliability of the method was satisfactory for all products evaluated.

Keywords: Protease activity; Milk; Azocasein; Validation

1. Introduction

Proteases are enzymes that degrade proteins. Raw milk may have proteolytic activity from endogenous or indigenous origin. Wherever they come from, proteases can cause unpleasant flavours and odours in milk (Fox, 1981; Veisseyre, 1988).

Several bacteria present in raw milk can produce proteases (Fox, 1981; Veisseyre, 1988). Among them, those from psychrotrophic microorganisms, which produce enzymes under refrigeration, cause the most serious problems (Cousin, 1982; Law, 1979). *Bacillus subtilis* is a psychrotrophic microorganism (Kohlmann, Nielsen, Steenson, & Ladisch, 1991) and secretes a thermo-resistant protease (Poffé & Mertens, 1988) which may cause proteolysis in pasteurized or sterilized milk (Law, Andrews, & Sharpe, 1977).

Diminution of thermal stability is a technological consequence of the presence of bacterial proteases in milk. On the other hand, these enzymes degrade caseins which implies losses in the yield of cheese and an increase of the nitrogen content of the whey (Gebre-Egziabher, Humbert, & Blankenagel, 1980; Veisseyre, 1988).

Several colourimetric methods have been described to detect and quantify protease activity (Rollema et al., 1989). Some of them measure the degradation of natural milk substrates due to the action of the proteolytic enzymes through the determination of the concentration of acid-soluble amino acids and peptides. The methods described by Hull (1947), Lowry, Rosebrough, Farr, and Randall (1951) and the tyrosine index (Juffs, 1973) quantify the acid-soluble protein fragments in the samples using the Folin reagent. Other methods are based on the measurement of amino groups freed during the hydrolysis of peptide bonds, such as use ninhydrin (Moore & Stein, 1954) or trinitrobenzene sulfonic acid (Fields, 1972), compounds that react with primary amino groups to give measurable coloured products. However, there are other types of assays which specifically and directly measure the proteolytic activity of the samples by means of the addition of an artificial substrate which, when hydrolyzed, releases coloured products. Azocasein (Charney & Tomarelli, 1947; Christen & Marshall, 1984) is one of the best known substrates. Moreover, it has been used to relate the growth of two microorganisms, *Pseudomonas fluorescens* M3/6 and *Pseudomonas fragi* K122, to the increase in the proteolytic activity in milk samples (Kohlmann et al., 1991).

It should be interesting to have a validated method for measuring the proteolytic activity in different media. In this study, proteolytic activity was quantified by a

* Corresponding author. Tel.: +34-97-3702593; fax: +34-97-3702596.

E-mail address: omartin@tecal.udl.es (O. Martín).

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colourimetric method using azocasein as artificial substrate in three different media: a model solution similar to milk ultrafiltrate (a fat- and protein-free solution), skim milk (fat-free) and whole milk. The method was evaluated in terms of linearity, sensitivity, precision and accuracy. Moreover, the influence of the fat and protein content of the samples on the reliability of the method was studied.

2. Materials and methods

2.1. Sample preparation

This study was performed on skim and whole milk, which were provided by Granja Castelló S.A. and in SMUF, the composition of which is shown in Table 1 (Jenness & Koops, 1962).

The protease from *Bacillus subtilis* was purchased in a liquid commercial form (Aldrich, Steinheim, Germany). According to the supplier's information, it contained 728 U/ml of protease activity. The enzyme activity was expressed in mU/ml after verifying that there was a linear correlation between enzyme activity in mU/ml (from commercial information) and enzyme concentration in mg/ml using the evaluated method.

Samples of whole milk (WM), skim milk (SM) or SMUF containing 10, 20, 30, 40 or 50 mU/ml were prepared to validate the method.

2.2. Enzyme determination

2.2.1. Reagents

Disodium hydrogenphosphate, sodium dihydrogenphosphate and trichloracetic acid were provided by Prolabo (Fontenay S/Bois, France) and the azocasein by Sigma Chemical Co. (St. Louis, MO, USA).

2.2.2. Solutions

A pH 7.2 buffer solution was prepared by mixing 36 ml of sodium hydrogenphosphate (5 mM) and 14 ml of sodium dihydrogenphosphate (5 mM) and diluting with distilled water to 1000 ml. This buffer was used to prepare a 1% azocasein solution. A 5% aqueous solution of trichloracetic acid was also required.

Table 1
Composition of simulated skim milk ultrafiltrate[a] (Jeness & Koops, 1962)

Lactose	$C_{12}H_{22}O_{11}\cdot H_2O$	50.00
Potassium phosphate	KH_2PO_4	1.58
Potassium citrate	$K_3C_6H_5O_7\cdot H_2O$	0.98
Potassium sulphate	K_2SO_4	0.18
Sodium citrate	$C_6H_5O_7\cdot 2H_2O$	1.79
Calcium chloride	$CaCl_2$	1.30
Magnesium citrate	$Mg_3C_{12}H_{10}O_{14}\cdot 9H_2O$	0.38
Potassium carbonate	K_2CO_3	0.3
Potassium chloride	KCl	1.00

[a] The values are expressed in g/l.

2.2.3. Determination of protease activity

The assay for proteolytic activity used in this case is based on that described by Christen and Marshall (1984). It involved combining 1 ml of the azocasein solution with 100 μl of the sample which contains the enzyme. The contents of the tubes were mixed and incubated at 35.5 °C for 15 min. The reaction was stopped by the addition of 2 ml of 5% TCA. The absorbance of the supernatant was read at 345 nm in a UV/Visible spectrophotometer (CECIL, CE 1021, England).

2.3. Validation of the analytical method

The analytical characteristics of the tested method in WM, SM or SMUF were validated to ensure the reliability of the results. Afterwards, the results obtained on SMUF or milk, with or without fat, were compared. The statistical treatments were performed with the Statgraphics plus Version 2.1. for Windows, statistical software (Statistical Graphics Co., Rockville, MD, USA, 1994–1996).

2.3.1. Validation

The method was validated in WM, SM and SMUF by linearity, sensitivity, precision and accuracy.

2.3.1.1. Linearity. The relation between the concentration of protease (dependent variable) and the absorbance at 345 nm (independent variable) defined linearity. It was evaluated by the analysis of variance of the regression equation. The experimental Fisher value (F_{exp}) was compared to its tabulated value (F_{tab}) for 1 and $n-2$ degrees of freedom (Steel & Torrie, 1980). If F_{exp} was higher than F_{tab} the existence of a linear relationship between variables ($P<0.001$) was accepted.

The correlation coefficient (R) was calculated by means of least-squares analysis, after three repetitions of six different concentrations (0, 10, 20, 30, 40 and 50 mU/ml) for each method and substrate. R-value indicated the relatively strong relationship between the variables.

Afterwards, the determination coefficient (R^2) was determined to show the extent of the total variability of the response that could be explained by the linear regression model.

2.3.1.2. Sensitivity. The detection limit (DL) and quantification limit (QL) were calculated from the calibration lines that defined linearity, using the Long and Winefordner criterion (Long & Winefordner, 1983). The

values of the slope of the calibration lines (b) and the standard error of the independent term of the regression (S_{b1}) were required. They were calculated according to the Eqs. (1) and (2).

$$\mathrm{DL} = \frac{3 \times S_{b1}}{b} \quad (1)$$

$$\mathrm{QL} = \frac{10 \times S_{b1}}{b} \quad (2)$$

2.3.1.3. Precision. The precision of the method indicated the degree of dispersion obtained with a series of determinations on the same sample.

Six measurements were performed for samples containing 50 mU/ml of the enzyme. The standard deviation and the relative standard deviation (RSD) were calculated, and the adequacy of the RSD for the Horwitz criterion (Horwitz, 1982) was evaluated. To consider RSD acceptable in terms of precision, the experimental values ($\mathrm{RSD_{exp}}$) should be lower than the RSD calculated throughout the Horwitz formula [Eq. (3)], where C is the average concentration of the analyte ($\mathrm{mU/ml \times 10^{-6}}$).

$$\mathrm{RSD} = 2^{(1-0.5\log C)} \quad (3)$$

2.3.1.4. Accuracy. This parameter showed the proximity between the experimental and theoretical values. The determination of this parameter was performed for each milk system by studying the recovery after standard addition procedure using two addition levels. The initial proteolytic activity of the samples was "non-detectable" and the study was carried out using concentrations of 10 and 50 mU protease/ml. Six determinations were carried out for each addition level and the% of recovery was calculated.

The homogeneity of variances of the measurements for each assayed level was verified by a Cochran test (Steel & Torrie, 1980).

The mean recoveries of each level were compared using a Student's t-test. It was carried out by comparing the experimental t-value (t_{exp}) to the tabulated one (t_{tab}) for ($n-1$) degrees of freedom (Steel & Torrie, 1980). Therefore, an average value of both levels could be considered when t_{exp} was lower than t_{tab}.

2.3.2. Influence of the medium on the reliability of the method

To compare the reliability of the method when performed in different media, in terms of linearity, sensitivity and recovery, first a variance analysis was performed to study the influence of the different media on each evaluated parameter. Afterwards, a comparison procedure was performed to find significant differences among the mean values obtained for the different milk media systems. The method currently used to discriminate among the means was Fisher's least significant difference (LSD) procedure ($P < 0.05$). Results of linearity were compared for three calibration lines with their respective r-values. The DL and QL were used to compare the sensitivity of the method for each medium. Finally, to carry out the comparison test on the accuracy, all the values of recovery of each set of analysis were considered.

3. Results

3.1. Linearity

Absorbance responses to the enzymatic activity of 0–50 mU/ml of protease in all the media were linear (Table 2). Least-squares analysis lead to correlation coefficients $r > 0.991$ for both skim and whole milk and 0.995 for SMUF ($P < 0.001$; Table 2). The coefficients of determination (r^2) were higher than 98.2% for milk and 99.0% for SMUF (Table 2).

3.2. Sensitivity

The values of DL and QL are shown in Table 3 where it can be observed that limits were lower when the

Table 2
Linearity parameters of the calibration lines for the evaluated method to determine protease activity in whole (WM), skim milk (SM) and simulated milk ultrafiltrate (SMUF)

Sample	Calibration line	R	R^2	F_{exp}[a]	P[b]
WM	$Y = 0.0086\,x + 0.0917$	0.991	0.982	799.83	<0.001
SM	$Y = 0.015\,x + 0.034$	0.991	0.982	861.27	<0.001
SMUF	$Y = 0.014\,x + 0.128$	0.995	0.990	1602.48	<0.001

[a] Statistic F-Fisher calculated experimentally, $F_{\mathrm{tab\ (1,16,0.001)}} = 16.12$.
[b] Significance level.

Table 3
Detection and quantification limits calculated throughout Long and Winefordner criterion (1983) for the evaluated method to determine protease activity in whole (WM), skim milk (SM) or simulated milk ultrafiltrate (SMUF)

Sample	Slope[a]	Standard error[b]	DL[c] (mU/ml)	QL[d] (mU/ml)
WM	0.008	0.009	3.26	10.9
SM	0.015	0.015	3.20	10.7
SMUF	0.014	0.011	2.29	7.64

[a] Slope of the calibration line.
[b] Standard error of the intercept point of the calibration line.
[c] Detection limit.
[d] Quantification limit.

method was carried out in SMUF than in milk. Minimum values of DL and QL of 3.20 and 10.7 mU/ml were found when the determinations were performed in skim milk, which were similar to that obtained in whole milk, whereas for SMUF, DL and QL results were 2.29 and 7.64 mU/ml.

3.3. Precision

Table 4 shows that relative standard deviations (RSD) of less than 8% were obtained in all systems and that all RSD were satisfactory according to the Horwitz criterion (Horwitz, 1982; Table 4).

3.4. Accuracy

The homogeneity of variances of the recovery was verified through a Cochran test (Table 5). The Student test showed that the recovery of the proteolytic activity did not depend on the enzyme concentration (Table 5). Therefore, the final recovery was the average of the results obtained in both levels of addition for each product (Table 5). Moreover, all the products led to a recovery similar to the theoretical 100% (Table 5).

Table 4
Precision of the assayed method for the determination of protease activity in whole (WM), skim milk (SM) and simulated milk ultrafiltrate (SMUF) and acceptable values of relative standard deviation (RSD) through Horwitz criterion

Sample	Activity ± DS[a] (mU/ml)	RSD (%)[b]	$RSD_{Horwitz}$
WM	50.3 ± 0.57	1.14	9.9
SM	47.8 ± 3.82	7.99	8.9
SMUF	50.8 ± 2.48	4.89	9.6

[a] Mean activity ± standard deviation in mU/ml.
[b] Acceptable RSD value through HORWITZ criterion.

Table 5
Recovery of the evaluated method to determine protease activity in whole (WM), skim milk (SM) and simulated milk ultrafiltrate (SMUF)

Sample	Recovery (%)		Cochran test[c]	Student test-L[d]	Mean recovery	Student test-100[e]
	Level I[a]	Level II[b]				
WM	103 ± 4	105 ± 2	0.79	1.82	104 ± 3	4.05
SM	103 ± 10	95 ± 8	0.76	1.48	99 ± 9	0.21
SMUF	100 ± 1	102 ± 1	0.70	1.44	101 ± 3	1.25

[a] Addition level I: 10 mU/ml.
[b] Addition level II: 50 mU/ml.
[c] Cochran test, $C_{tab\ (2,5,0.01)} = 0.877$.
[d] Test to determine the difference among the recoveries obtained in levels I and II, $t_{tab} = 6.869$.
[e] Test to determine the differences among the mean revovery and the theoretical 100%, $t_{tab} = 4.437$.

4. Discussion

The evaluated method when performed in SMUF or milk with different fat content was significantly linear in the range of 0–50 mU/ml. The correlation coefficients of the calibration lines were determined to compare the goodness of the linearity. No differences were observed for the *r*-values obtained when carrying out the method in the different milk systems ($P < 0.05$).

The sensitivity was studied throughout the parameters DL and QL and the least values were, respectively, 2.29 and 7.64 mU/ml. After a comparison of the results of sensitivity, it was observed that the DL and QL obtained did not depend significantly on the kind of media. In terms of precision, the method fulfilled the Horwitz criterion in all cases. So, it may be considered precise for use in samples with different protein or fat contents. The accuracy was studied through the recovery of two levels of addition. Results did not depend on the protease content of the samples; thus the recovery could be taken as the average of the recoveries obtained at both levels and was always similar to the theoretical 100%.

This method is useful for determining the proteolytic activity in different media and its goodness does not depend on the fat or protein content of the sample, since the results obtained on the evaluated parameters were similar in SMUF (a fat and protein free solution), skim (fat free) and whole milk. Milk components do not interfere in the reliability of the results. So, the method is good enough to determine the proteolytic activity in all these different products.

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References

Charney, J., & Tomarelli, R. M. (1947). A colourimetric method for the determination of proteolytic activity of duodenal juice. *Journal of Biological Chemistry*, *171*, 501–505.

Christen, G. L., & Marshall, R. T. (1984). Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media. *Journal of Dairy Science*, *67*, 1680–1687.

Cousin, M. A. (1982). Presence and activity of psycrotrophic microorganisms in milk and dairy products: a review. *Journal of Food Protection*, *45*, 172–207.

Fields, R. (1972). The rapid determination of amino groups with TNBS. *Methods in Enzymology*, *25*, 464–468.

Fox, P. F. (1981). Proteinases in dairy technology. *Netherlands Milk and Dairy Journal*, *35*, 233–253.

Gebre-Egziabher, A., Humbert, E. S., & Blankenagel, G. (1980). Hydrolisis of milk proteins by microbial enzymes. *Journal of Food Protection*, *43*(9), 709–712.

Horwitz, W. (1982). Evaluation of analytical methods used for regulation of foods and drugs. *Analytical Chemistry*, *54*, 67–76.

Hull, M. E. (1947). Studies on milk proteins II. Colourimetric determination of the partial hydrolisis of the proteins in milk. *Journal of Dairy Science*, *30*, 881–884.

Jenness, R., & Koops, J. (1962). Preparation of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk and Dairy Journal*, *16*(3), 153–164.

Juffs, H. S. (1973). Proteolysis detection in milk. Interpretation of tyrosine value data for raw milk supplies in relation to natural variation, bacterial counts and other factors. *Journal of Dairy Research*, *40*, 371–381.

Kohlmann, K. L., Nielsen, S. S., Steenson, L. R., & Ladisch, M. R. (1991). Production of proteases by psychrotrophic microorganisms. *Journal of Dairy Science*, *74*, 3275–3283.

Law, B. A. (1979). Enzymes of psychrotrophic bacteria and their effects on milk and milk products. *Journal of Dairy Research*, *46*, 573–588.

Law, B. A., Andrews, A. T., & Sharpe, M. E. (1977). Gelation of UHT sterilized milk by proteases from a strain of *Pseudomonas fluorescens* isolated from raw milk. *Journal of Dairy Research*, *44*, 145–178.

Long, G. L., & Winefordner, J. D. (1983). Limit of detection. A closer look at the IUPAC definition. *Analytical Chemistry*, *55*, 712–724.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, *163*, 265–275.

Moore, S., & Stein, W. H. (1954). A modified ninhydrin reagen for the photometric determination of amino acids and related compounds. *Journal of Biological Chemistry*, *211*, 907–913.

Poffé, R., & Mertens, W. (1988). Heat stable proteases of psychrotrophic bacteria isolated from cooled raw milk. *Applied Microbiology and Biotechnology*, *27*, 437–442.

Rollema, H. S., Mckellar, R. C., Sørhaugh, T., Suhren, G., Zadow, J. G., Law, B. A., Poll, J. K., Stepaniak, L., & Vagias, G. (1989). Comparison of different methods for the detection of bacterial proteolysis enzymes in milk. *Milchwissenschaft*, *44*, 491–496.

Steel, R. G. D., & Torrie, J. H. (1980). *Principles and procedures of statistics. A biometrical approach* (2nd ed.). New York, NY: McGraw Hill.

Veisseyre, R. (1988). *Lactología técnica*. Zaragoza: Acribia.