Problems with Detection of Proteolytic Microorganisms and Their Undesirable Activities in Milk

Irena NĚMEČKOVÁ[1], Marta PECHAČOVÁ[2] and Petr ROUBAL[1]

[1]Dairy Research Institute Ltd., Prague, Czech Republic; [2]MILCOM Inc., Prague, Czech Republic

Abstract: Occurrence of proteolytic enzymes in milk is often associated with technological problems and sensory, rheological and functional defects of final dairy products. Thus, the simple, cost-effective and available laboratory method for evaluation of undesirable proteolysis risk is needed. In our work we have tested cultivation plate methods and chemical methods (formol titration, ammonium reflectometric determination, the Kjeldahl method, the agar-well diffusion assay and spectrofotometry after cleavage of azo-casein) to choose the proper ones which can provide information on undesirable proteolytic changes especially in raw milk. Although the microbiological analyses cannot detect enzymes indigenous to milk, but only the quantity of producers of microbial enzymes, they seem to be the most acceptable, particularly usage of the Glucose-Trypton-Yeast Extract agar with 10% vol. of sterile milk added before pouring onto plates (incubation at 30°C for 72 h). The chemical methods are not sensitive enough to analyse the real milk samples.

Keywords: proteolysis; spoilage risk assessment; cultivation method; simple chemical methods; raw milk; dairy products; *Bacillus cereus*

Proteolytic enzymes in milk can be categorised according to their origin as indigenous or microbial (extracellular, intracellular or periplasmatic) (Shamsuzzaman & McKellar 1987).

Milk contains a number of indigenous proteinases, of which plasmin (EC 3.4.21.7) is the most important. Complex plasmin system comprises plasmin, plasminogen, plasminogen activators, plasmin inhibitors and inhibitors of plasminogen activators. In milk, plasminogen, plasmin and plasminogen activators are associated with the casein micelles while plasmin inhibitors and inhibitors of plasminogen activators are in the serum phase. Plasmin has trypsin-like specificity and acts on caseins in the order $\beta \approx \alpha_{s2} >> \alpha_{s1}$, while κ-casein appears to be resistant (Upadhyay *et al.* 2004). Although optimal conditions for plasmin activity are about 37°C and pH 7.5, it can evoke proteolytic changes during raw milk storage at 4°C, as well (Ma *et al*. 2003).

In the mastitis cow's milk there is an increase in the count of somatic cells, which contain many active proteinases, including cathepsins B, D, G, H, L and elastase. The presence of cathepsins D (EC 3.4.23.5) and B (EC 3.4.22.1) in milk was confirmed (Upadhyay *et al.* 2004).

Cathepsin D cleaves α_{s1} and β-casein in a similar way as the rennet, liberates para-κ-casein from κ-casein and is able to coagulate milk at pH 5.0–6.5, although its pH optimum is about 4.0 (Hurley *et al*. 2000).

Cathepsin B has higher pH optimum (about 6.0) than cathepsin D but the same optimal temperature (about 37°C). It cleaves α_{s1} and β-casein and is able to damage cheese texture by hydrolysis of Phe_{23}-Phe_{24} bond in α_{s1}-casein (Considine *et al*. 2004).

Microbial proteolytic enzymes producers belong to various geni, e.g. *Pseudomonas, Bacillus, Clostridium, Proteus, Escherichia, Micrococcus,*

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Microbacterium, Flavobacterium, Chryseobacterium, etc. while the first two ones are the most important.

Bacilli produce broad spectrum of proteolytic enzymes that vary in their quantity and relative ratio from strain to strain. Thus, there is only a weak correlation between counts of aerobic spore-formers and risk of spoilage (Chen *et al*. 2004). Most extracellular proteinases are synthesised at the end of exponential phase and during stationary phase and sporulation. Proteolytic changes manifest themselves by increase in non-protein nitrogen concentration and by para-κ-casein formation accompanied by casein micelles destabilisation and milk coagulation (Chen *et al.* 2003). Bacillary proteinases usually have a pH optimum in alkaline zone (Chen *et al*. 2004) and a temperature optimum at 30–37°C (Braun *et al*. 1999).

In contrast to bacilli, pseudomonades usually produce only one type of protease (Chen *et al.* 2003), mainly at the end of exponential phase and during stationary growth phase, when their density reaches 10^7–10^8 CFU/ml (Grieve & Kitchen 1985). However, at low temperature (about 2°C) proteolytic enzymes are detectable already at density 10^4–10^5 CFU/ml and their concentration is higher than at temperature over 20°C (Braun & Sutherland 2003). *Pseudomonas* spp. affect especially κ-casein, casein micelles and colloid calcium phosphate (Guinot-Thomas *et al*. 1995). Their proteinases usually have optimal conditions at temperature 17.5–30°C and pH 6.5–7.0 (Braun & Sutherland 2003).

Spoilage proteolytic enzymes play an important role in shelf-life and sensory quality of many dairy products. Pasteurised milk sensory properties may deteriorate if the raw milk contained about 10^5–10^6 CFU/ml of psychrotrophs, while heat exchanger fouling and shelf-life shortening is expected at higher initial densities (Sørhang & Stepaniak 1997). Pseudomonades are known as the most common post-pasteurisation contamination, as well (McPhee & Griffiths 2003). However, *Bacillus cereus* seems to be the most important micro-organism limiting pasteurised milk shelf-life (Te Giffel *et al*. 2000).

Proteolytic enzymes present in raw milk can partially survive heat treatment and cause defects of UHT milk, e.g. off-flavor formation (bitterness, astringency or fustiness) (Santos *et al.* 2003) or consistency defects (thickening, coagulation or gelation) (Celestino *et al.* 1997).

These enzymes survive milk powder manufacturing, as well. Although low water activity inhibits microbial growth, enzymatic activity persists and causes sensory and functional defects (Chen *et al.* 2004). Lowered thermo-stability and increased foam-formation of reconstituted milk can appear in consequence of density 10^6–10^7 CFU/ml of psychrotrophs in raw milk (Sørhang & Stepaniak 1997).

Although undesirable proteolytic changes in fermented milk are not as frequent as defects due to improper fermentation control, they can lead to off-flavor formation, especially bittering (Mistry 2001).

Proteolysis in milk can be studied by two approaches – quantification of proteolytic changes and determination of proteolytic enzymes activity. Both of them have particular positive and negative aspects. Methods for detection of milk proteins degradation products (electrophoresis, the Kjeldahl method, etc.) are reliable but time-consuming and inconvenient for manufacture use, as well. On the contrary, spectrofotometrical methods (of the both approaches mentioned above) are simple and quick, but their sensitivity is relatively low.

The most wide-spread methods for enzymes activity determination are spectrofotometrical ones. Because of milk contains low enzymes concentration and high protein concentration, there is a considerable risk of distorted data, even in case of higher enzyme specificity for chromogenic substrate than for milk proteins present in milk. Thus, determination of enzymatic activity is more suitable for isolated enzymes characterisation (Chen *et al*. 2003). There is a view of methods in Tables 1 and 2. Special consideration should be given to chromatographic method (reversed-phase HPLC) optimised by Datta and Deeth (2003) that enables evaluation whether UHT milk defects were caused by microbial or indigenous enzymes according to the presence of specific peptide peaks in sample filtrate after precipitation by trichloroacetic acid.

The main goal of this work is to recommend to dairy-plant laboratories a simple, cheap, robust and available method for evaluation of undesirable proteolytic changes especially in raw milk.

MATERIAL AND METHODS

Micro-organisms. *Bacillus cereus* SPA 12 (isolated from dairy industry surface scum), *Bacillus licheni-*

Table 1. Methods for detection of proteolytic changes

Method type	Examples	References
Electrophoresis	Electrophoresis in starch gel (SGE)	Juffs (1975)
	Electrophoresis in polyacrylamide gel (PAGE)	Andrews (1982)
	Electrophoresis in polyacrylamide gel after urea addition (UREA-PAGE)	Kelly & Foley (1997)
	Electrophoresis in polyacrylamide gel after sodium dodecyl-sulphate addition (SDS-PAGE)	Grieve & Kitchen (1985)
Liquid chromatography	High performance liquid chromatography on reversed phase (RP-HPLC) with UV detection	Considine *et al.* (2004)
Spectrofotometry	Tyrosine and tryptophan reaction with Folin-Ciocalteu reagent	AlKanhal *et al.* (1994)
	Free amino-groups reaction with nitro-benzene-sulfonic acid	AlKanhal *et al.* (1994)
	α-amino-groups reaction with fluorescamine	Chen *et al.* (2003)
	α-amino-groups reaction with tri-nitro-benzene-sulfonic acid (TNBS)	Chen *et al.* (2003)
	TCA-soluble filtrate reaction with o-phthaldialdehyde (OPA)	Matselis & Roussis (1998)
Kjeldahl method	Determination of amino-acids and peptides soluble in 10% tri-chloro-acetic acid	Juffs (1975)
	Determination of non-casein nitrogen soluble at pH 4.6	Valero *et al.* (2001)
	Casein and total proteins ratio	Ma *et al.* (2003)
Titration	Formol titration	Juffs (1975)

formis SPA 9 (isolated from raw cow milk) and *Pseudomonas aeruginosa* CCM 3955 (Czech Collection of Micro-organisms, Brno, Czech Republic) were maintained on the Glucose-Tryptone-Yeast Extract (GTY) agar (Merck KGaA, Darmstadt, Germany) after cultivation at 30°C for 18 hours.

Model samples preparation. Sterile skimmed milk inoculated with *B. cereus* and incubated at 30°C for 24 h (precipitation took place during this period) and non-inoculated control sample were used for the first search for suitable chemical method. These two samples represented upper and lower limit of considered measuring range at industry applications. Thus, chemical methods, selected for further testing (the same experiment with *B. licheniformis* or *P. aeruginosa*, and then with real samples), should provide differences between these samples that are significant and enable further finer resolution.

Counts of micro-organisms present in prepared samples were evaluated on the GTY agar (Merck KGaA, Darmstadt, Germany) after incubation at 30°C for 72 h (EN ISO (2003), No. 4833).

If not stated otherwise, all samples were prepared in duplicate and analysed twice (totally $n = 4$).

Ammonium determination. Commercial Ammonium Test (Merck KGaA, Darmstadt, Germany) for portable reflectometer RQflex 2 (Merck KGaA, Darmstadt, Germany) was used for hundredfold diluted sample analyses according to manufacturer's instruction.

Formol titration. Analyses were performed according to methodology described by Juffs (1975).

Kjeldahl method. Total nitrogen content, non-protein nitrogen content and protein nitrogen content were determined according to standards EN ISO (2003), No. 8968-1, EN ISO (2002), No. 8968-4 and EN ISO (2002), No. 8968-5, resp.

Agar-well diffusion assay. Method recommended by Braun *et al.* (1999) for isolated enzymes in buffer was modified for milk analyses. To prevent micro-organisms from growing, samples should be sterilised (without heating that could partially inactivate enzymes). Milk centrifugation at 7000 g for 15 min at room temperature to remove the largest particles and then microfiltration via sterile filters (pores 0.2 μm, Minisart, Sartorius AG, Goettingen, Germany) were approved as the sample pre-treatment procedure.

Table 2. Methods for determination of proteolytic enzymes activity

Method type	Examples	References
Spectrofotometry	Reaction with N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl-coumarine	Kelly & Foley (1997)
	Reaction with N-succinyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (Suc-AAPF-pNA)	
	Reaction with bovine serum albumin (BSA)	
	Reaction with D-valyl-L-leucyl-L-lysine p-nitroanilide dihydrochloride	Chen *et al.* (2004)
	Reaction with azo-casein	Shamsuzzaman & McKellar (1987) Guinot-Thomas *et al.* (1995) Bendicho *et al.* (2002)
Fluorimetry	Reaction with fluorescein-thiocarbamoyl-β-casein (FTC-β-CN)	Chen *et al.* (2004)
Luminometry	Bioluminescent method with luciferase	Reybroeck (2000)
Immunomethods	Enzyme-linked immuno-sorbent assay (ELISA)	Chen *et al.* (2003)
Agar plate methods	Agar-well diffusion assay	Braun *et al.* (1999)

Diameter of cleared zones was measured at wells of diameter 9 mm (contained 30 µl of sample) in the GTY agar with 10% vol. of sterile milk added before pouring onto plates (GTY-M). Incubation proceeded at 30°C for 72 h, i.e. at the same temperature as used for preparation of sample destabilised by micro-organisms.

Spectrofotometrical method with azo-casein. Method used by Bendicho *et al.* (2002) was modified. 0.2 ml of milk sample was added to 2 ml of 1% azo-casein solution in 0.1 mol/l TRIS.HCl buffer pH 7.5 and incubated at 37°C for 1 hour. Reaction was stopped by addition of 5% trichloracetic acid in volume of 4 ml and precipitate was removed by filtration (pores 0.2 µm, Minisart, Sartorius AG, Goettingen, Germany). Absorbance was measured at wave length 346 nm.

Comparison of cultivation plate methods for proteolytic micro-organisms determination. Proteolytic micro-organisms were determined in 25 raw milk samples on tested agar media – the Calcium Caseinate (CC) agar (Merck KGaA, Darmstadt, Germany) and the GTY-M agar. Plates were incubated at 30°C for 72 h and then the colonies with cleared zones were counted. Total counts on the GTY-M agar were compared with the total mesophilic count according to EN ISO (2003), No. 4833, as well. Differences between results measured for each sample were expressed graphically to find out whether there is any systematic

Table 3. Data on sample analyses by different chemical methods (n = 4)

Method	Analyte	Sterile sample	Sample precipitated by *Bacillus cereus*
Reflectometry	ammonium	0.10 ± 0.03 g/l	0.23 ± 0.07 g/l
Formol titration	proteins	3.30 ± 0.39 g/100 ml	3.85 ± 0.46 g/100 ml
Kjeldahl method	total nitrogen	0.518 ± 0.047 g/100 g	0.538 ± 0.052 g/100 g
	non-protein nitrogen	0.030 ± 0.004 g/100 g	0.103 ± 0.011 g/100 g
	protein nitrogen	0.488 ± 0.050 g/100 g	0.434 ± 0.046 g/100 g
Agar-well diffusion assay	proteases (as diameter of cleared zones under the method conditions)	Non-detectable	24 ± 2 mm
Spectrofotometry after reaction with azo-casein	proteases (as absorbance at 346 nm)	0.002 ± 0.002	0.018 ± 0.006

deviation. Two-sample pair *t*-test was performed and Pearson's correlation coefficient was evaluated by MS Excel 2000.

RESULTS AND DISCUSSION

B. cereus was inoculated into sterile milk to form density 5.3 ± 0.3 log CFU/ml and cultivated at 30°C for 24 h to form density 8.0 ± 0.3 log CFU/ml and precipitate milk. This sample and non-inoculated control sample were used for the first selection of suitable chemical method. Data are shown in Table 3.

It is obvious that ammonium reflectometrical determination, formol titration and total nitrogen and protein nitrogen determination by the Kjeldahl method are not able to differentiate both between analysed samples and among more closely graduated real samples. According to Juffs (1975), formol titration did not appear to have any application in the screening of cold-stored raw milk with total bacterial counts lower than 7 log CFU/ml, as well. Data on non-protein nitrogen are rather more favourable, but not as potential as expected. Furthermore, the Kjeldahl method is too time-consuming and labour-intensive to be used at dairy-plants laboratories.

Absorbances obtained by spectrofotometry after reaction with azo-casein were too low to be reliable, as well. Samples should be pre-treated to provide absorbances between 0.100 and 1.000 on instrument display, but both used and modified methods even with prolonged reaction time did not offer suitable results (data not shown). The least detection and quantification limits of the method, as published by Bendicho *et al.* (2002), were 2.29 mU/ml and 7.64 mU/ml of *Bacillus subtilis* protease added to milk, respectively, but these activities may be higher than those present in analysed samples.

Only the agar-well diffusion assay was selected for subsequent testing with other microbial strains. *B. licheniformis* was inoculated to form density 5.4 ± 0.3 log CFU/ml and cultivated at 30°C for 24 h to form density 7.8 ± 0.4 log CFU/ml. During this period no visible change of milk was observed and no cleared zones around the agar-wells, as well. (Plates were incubated at 25, 30 and 44°C for 72 h and 144 h to find out optimal conditions.)

Initial density of *P. aeruginosa* was 6.9 ± 0.2 log CFU/ml, density after incubation at 30°C for 24 h was 8.6 ± 0.4 log CFU/ml. During their growth, pseudomonades destabilised milk proteins which was approved by cleared supernatant of centrifuged sample. However, cleared zones around the agar-wells were observed only after incubation at 44°C for 144 hours. On the contrary, Braun and Sutherland (2003) observed optimal temperature for extracellular pseudomonades enzymes within the range 17.5–30°C.

Thus, it is evident that the agar-well diffusion assay is suitable only for some enzymes but not for the sum of proteases present in milk. Regarding to result mentioned above, chemical methods testing did not continue.

Cultivation methods for determination of proteolytic micro-organisms, as an important source

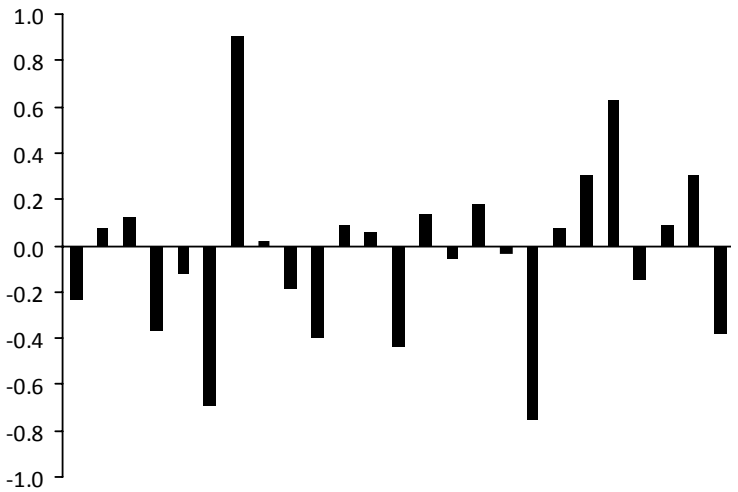

Figure 1. Differences between counts (log CFU/ml) of proteolytic micro-organisms determined on the GTY-M agar and on the CC agar after incubation at 30°C for 72 h in 25 raw milk samples (n = 2)

Figure 2. Differences between total counts (log CFU/ml) of micro-organisms determined on the GTY agar and on the GTY-M agar after incubation at 30°C for 72 h in 25 raw milk samples (n = 2)

of proteolytic activity in milk, were compared. As shown on Figure 1, there is no systematic deviation between results obtained after cultivation on the GTY-M agar and on the CC agar. All counted differences are less than 30% of the lower result which is a common deviation of cultivation analyses. Pearson's correlation coefficient is 0.8750 that means good correlation between the both methods.

It could be useful to assess both proteolytic micro-organisms percentage and total microbial contamination level in one step. Thus, total counts on the GTY-M agar were compared with the total mesophilic count on the GTY agar according to EN ISO (2003), No. 4833. There is no systematic deviation between the results obtained by these two methods (Figure 2). All counted differences are less than 10% of the lower result and Pearson's correlation coefficient is 0.9973. Although the GTY-M agar usage should be validated according to internationally accredited protocol, e.g. EN ISO (2003), No. 16140, it seems to be a useful tool for dairy plants laboratories routine analyses that could provide more information without increase in material costs or in time consumption in comparison with the reference method.

CONCLUSION

Milk samples – sterile milk and milk precipitated by *B. cereus* (final density 8.0 ± 0.3 log CFU/ml of *B. cereus*), and additionally samples with *P. aeruginosa* and *B. licheniformis* grown to density 8.6 ± 0.4 log CFU/ml and 7.8 ± 0.4 log CFU/ml, respectively, were used for the selection of method suitable for risk assessment of undesirable proteolytic changes in milk and dairy products. Formol titration, ammonium reflectometric determination, the agar-well diffusion assay, the Kjeldahl method and spectrofotometry after cleavage of chromogenic substrate azo-casein were tested. However, none of them provided sufficient difference between samples that could enable further finer resolution.

The question may be why the methods, used by other authors, did not approve themselves at this work. The reason could be in experiments methodology and goals. Chemical methods work well when used for monitoring of time-depending changes in milk samples with only one micro-organism or enzyme added to high concentration or for experiments with isolated pre-concentrated enzymes in synthetic media under the optimised conditions. On the contrary, proteolytic enzymes naturally present in milk constitute complex system with components of various concentration, location, optimal conditions and mode of action. Moreover, enzymes occur in milk in relatively low concentration and their diverse location disable pre-concentration. Nitrogen compounds composition is effected by number of factors other than proteolysis, e.g. cow's nutrition, breed or health. Thus, recognition of proteolytic changes in milk is too complicated task to be performed without special equipment.

Although microbiological analyses are unable to detect enzymes indigenous to milk, they seem to be a useful tool for dairy-plants laboratories. Usage of the GTY-M agar enables simultaneous estimation of total mesophilic contamination and percentage of proteolytic micro-organisms in one step. Result interpretation is the goal of future work. So far, the ratio of the total mesophilic count and the psychrotrophic bacteria count (including most of proteolytic micro-organisms in raw milk) was evaluated. The EU standards for the top quality milk require that the total mesophilic count and the psychrotrophic bacteria count shall not exceed 30 000 CFU/ml and 5000 CFU/ml, respectively, which corresponds to the ratio 6/1. Changes in the ratio are regarding as a frequent cause of the current unexplained problems in milk processing (Cempírková 2002).

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Corresponding author:

Ing. Irena Němečková, Výzkumný ústav mlékárenský, s.r.o., Ke Dvoru 12a, 160 00 Praha 6, Česká republika
tel.: + 420 235 354 551, e-mail: nemeckova@milcom-as.cz