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Original article

Studies on the thermal sensitivity of γ-glutamyl transpeptidase measured with a modified test procedure and compared with that of alkaline phosphatase and lactoperoxidase in milk

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Abstract – The milk enzyme γ-glutamyl transpeptidase (γ-GT) might be a useful indicator for monitoring the various conditions of milk pasteurisation. In the present work, γ-GT activity was measured using Clarifying Reagent® to facilitate spectrophotometric measurement. The proposed method exhibited a good sensitivity (7.1 mU·mL^{-1}) and a satisfying repeatability with a CV of 3% (n = 20). It was well correlated with the reference method for γ-GT activity measurement in 40 bovine milk samples (r = 0.98). The stability of γ-GT to milk heat treatments was compared with that of alkaline phosphatase (ALP) and lactoperoxidase. The thermal denaturation characteristics of γ-GT were very close to those of lactoperoxidase. γ-GT and ALP were of interest to control the label "made from raw milk" in Camembert cheese. This method could be suitable for routine quality control and assessment of mild heat treatment.

Bovine milk / cheese / γ-glutamyl transpeptidase / alkaline phosphatase / lactoperoxidase / heat treatment

Résumé – **Étude de la thermosensibilité de la γ-glutamyl transpeptidase, mesurée à l'aide d'une méthode modifiée faisant appel à la transparisation, comparée à celles de la phosphatase alcaline et de la lactoperoxydase dans le lait.** La γ-glutamyl transpeptidase (γ-GT) est une enzyme du lait qui pourrait être un indicateur utile pour la surveillance des conditions de pasteurisation du lait. Dans la méthode proposée, l'activité de la γ-GT a été mesurée en utilisant le Réactif de Transparisation® pour faciliter la mesure spectrophotométrique. La méthode proposée présente une bonne sensibilité (7,1 mU·mL^{-1}) et une répétabilité satisfaisante avec un CV de 3 % (n = 20). Les résultats de la mesure d'activité γ-GT de 40 échantillons de lait bovin obtenus par la méthode proposée sont en

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bonne corrélation avec ceux obtenus par la méthode de référence (r = 0,98). La stabilité de la γ-GT dans le lait soumis à différents traitements thermiques a été comparée à celles de la phosphatase alcaline (ALP) et de la lactoperoxydase. Les caractéristiques de la dénaturation thermique de la γ-GT étaient très proches de celles de la lactoperoxydase. Les activités γ-GT et ALP résiduelles pourraient être intéressantes pour le contrôle de l'appellation « au lait cru » du camembert. L'utilisation de cette méthode serait possible en analyse de routine pour le contrôle de la qualité ainsi que pour l'évaluation des traitements thermiques modérés.

Lait / fromage / γ-glutamyl transpeptidase / phosphatase alcaline / lactoperoxydase / traitement thermique

1. INTRODUCTION

Heat treatment is essential for hygienic safety and for extending the shelf life of milk. Due to their varying thermal resistance, some milk enzymes can be used as indicators of the severity or effectiveness of heat treatment of milk and milk products [2, 7, 16]. Enzyme assays, though limited to heat treatment up to the mildest of ultra high temperature (UHT) treatment, have a potential advantage in providing screening methods that are faster, simpler, and often less expensive than chemical analysis [30]. Measurement of peroxidase, catalase and alkaline phosphatase activities have been widely used to evaluate the degree and efficiency of mild heat treatments [5].

Alkaline phosphatase (ALP) (EC 3.1.3.1) has been used for many years as an indicator of adequate pasteurisation of milk [21, 22]. Enzyme activity is optimal at alkaline pH (9.8–9.9). ALP, which is relatively heat-sensitive, is completely inactivated by heating above 70 °C for 15 s [7]; its thermal resistance is only slightly higher than that of non-spore-forming pathogens such as *Mycobacterium tuberculosis* [6, 30]. However, some problems are related to the reversibility of its inactivation under certain conditions [16, 18, 21].

Lactoperoxidase (LPO) (EC 1.11.1.7) is one of the most thermo-stable enzymes in milk and has been used in the Storch test as an indicator of flash pasteurization [7, 26]. Monget and Laviolette [17] report that the enzyme is completely inactivated by heating for 20 s at 80 °C. Griffiths [7] indicates that heating milk at 80 °C for 15 s only reduces LPO activity by about 40% in laboratory studies, while a complete inactivation of the enzyme is obtained after treatment at 78 °C for 15 s, or 80 °C for 5 s, with a plate heat exchanger.

γ-Glutamyl transpeptidase or γ-glutamyl transferase (γ-GT) (EC 2.3.2.2) of mammalian tissue is a membrane-bound glycoprotein consisting of two subunits of 57.0 and 25.5 kg·mol^{-1} [4, 28]. Milk γ-GT is a membrane-bound enzyme [3, 4]. It catalyses the transfer of γ-glutamyl residues from glutathione, or other γ-glutamyl compounds, to amino acids or peptides [2] and plays an important role in the transport of amino acids to the mammary glands where they are used for milk protein synthesis. Glutathione, which is the natural substrate of the γ-GT, was initially used to measure the activity of this enzyme in serum.

Various synthetic substrates such as D, L-γ-glutamyl aminoproprionitril, N-(D, L-γ-glutamyl) aniline and L-γ-glutamyl-α-naphthylamide have been proposed [24]. Only L-γ-glutamyl-*p*-nitroanilide has been retained for routine use [20, 24]. γ-GT activity is generally measured with L-γ-glutamyl-*p*-nitroanilide as the donor of the γ-glutamyl moiety and glycylglycine as the acceptor substrate.

γ-GT activity seems to be independent of seasonal variations [16, 21]. Only little variations have been observed over an experimental period of 301 d with either

whole or skim milk. The average values of enzymatic activity were 4.30 ± 0.30 and 3.11 ± 0.18 U·mL^{-1} in whole milk and skim milk, respectively [16].

Clarifying Reagent® [14] is a mixture of organic solvents and detergents with an apparent pH >13 and a very low absorbance between 340 and 800 nm. Clarifying Reagent® renders casein micelles and fat globules soluble and allows direct spectrophotometric measurements to be made without the preliminary precipitation and filtration steps needed in many protocols. Many applications of milk clarification, such as the chemical measurement of NH_2^- [10] and SH-groups [8] or enzymatic measurement of N-acetyl-ß-D-glucosaminidase [11] or lipase [12] activities, can be used in routine analysis.

This study was undertaken to develop a rapid and easy colorimetric method for γ-GT measurement that avoids the precipitation and filtration steps usually required for turbidity elimination before spectrophotometric measurement. The potentiality of γ-GT to be a marker for heat treatment was investigated by studying its stability in the range 50–80 °C in comparison with two other endogenous milk enzymes (ALP and LPO) already used for the evaluation of milk pasteurisation [22]. In addition, the residual activities of the three enzymes were determined in different samples of Camembert cheese.

2. MATERIALS AND METHODS

2.1. Materials

Commercial bovine γ-glutamyl transpeptidase (EC 2.3.2.2), lactoperoxidase (EC 1.11.1.7), *p*-phenylenediamine and diethanolamine were obtained from Sigma Chemical Co (St Louis, MO, USA). Guaiacol, *p*-nitrophenylphosphate and other chemical reagents were from Merck (Darmstadt, Germany). Rothenfusser reagent contained 30 mL 18.4 mmol·L^{-1} *p*-phenylenediamine hydrochloride in distilled water mixed with 270 mL 53.7 mmol·L^{-1} guaiacol in 96% ethanol. Clarifying Reagent® was from Prolabo (Fontenay-sous-Bois, France). Milk samples were from individual fresh raw milks and commercial pasteurised milks. Camembert cheese samples were from local markets.

Absorbance was measured with a Shimadzu UV 2100 spectrophotometer (Roucaire, Courtabœuf, France).

For the cheese samples' preparation, aliquots of 5 g of grated cheese were suspended in a final volume of 100 mL with distilled water. This suspension was homogenised for 1 min in a stomacher. Samples were aliquoted and frozen at –18 °C until analysis.

2.2. Enzymatic measurement

2.2.1. γ-Glutamyl transpeptidase assay

Enzymatic activity was measured in the milk samples by the proposed protocol using the Clarifying Reagent® and by the method reported by Baumrucker [3] and modified by Andrews et al. [2] and Patel and Wilbey [21]. Both methods used L-γ-glutamyl-*p*-nitroanilide (γ-GPNA) and glycylglycine as donor and acceptor substrates, respectively.

In the proposed protocol, enzymatic reaction occurred at pH 8.5 and liberated *p*-nitroaniline, a yellow compound which strongly absorbs between 350–420 nm [20]. Absorbance was measured at 410 nm after addition of the Clarifying Reagent® which stopped the enzymatic reaction and clarified the sample. The following protocol was adopted after optimisation of experimental conditions for the sensitivity and stability of the reaction.

In a test tube, 2 mL of 0.1 mol·L^{-1} Tris-HCl buffer, pH 8.5, containing 4 mmol·L^{-1} γ-GPNA and 40 mmol·L^{-1} glycylglycine were preheated at 37 °C for 2 min. The

enzymatic reaction was started with the addition of 100 µL of raw or pasteurised milk or of cheese preparation. The milk sample was diluted with inactive boiled milk if necessary; no dilution was required for the cheese suspension. The reaction mixture was gently mixed and incubated at 37 °C for 15 min (milk) or 30 min (cheese). Clarifying Reagent® (2 mL) was then added and the tube was vigorously shaken and incubated for 2 min at 37 °C for liquid milk, and 5 min for the cheese suspension. Absorbance at 410 nm of the clarified mixture was measured against a blank tube within 10 min. The blank tube contained all reagents and boiled milk instead of a raw or pasteurised milk sample. All assays were in triplicate. The quantity of *p*-nitroaniline formed was calculated from a standard curve which was established with increasing amounts of *p*-nitroaniline (0.01 to 0.15 mmol·L^{-1} per assay) in 2 mL 0.1 mol·L^{-1} Tris-HCl buffer, pH 8.5 containing 40 mmol·L^{-1} glycylglycine in the presence of 100 µL of boiled milk and 2 mL of the Clarifying Reagent®. One unit of activity (U) is defined as the quantity of enzyme that released 1 µmol of *p*-nitroaniline per min at 37 °C and pH 8.5.

The results obtained with the proposed method were compared with those of the method of Patel and Wilbey [21]. The assays were carried out in duplicate at 37 °C using 25 µL of milk sample and 2 mL of buffer-substrate solution (2 mmol·L^{-1} γ-GPNA and 20 mmol·L^{-1} glycylglycine in 0.1 mol·L^{-1} Tris-HCl buffer, pH 8.0). Absorbance was recorded at 410 nm over a period of 5 min.

2.2.2. Lactoperoxidase assay

Oxidation of guaiacol and of *p*-phenylenediamine by lactoperoxidase was performed in a piperazine buffer at pH 6.7 in the presence of hydrogen peroxide using the modified method of Guingamp et al. [9]. The produced purple compounds were assessed by measuring absorbance at 560 nm after addition of the Clarifying Reagent® to stop the enzymatic reaction and clarify the mixture. 1 mL 0.025 mol·L^{-1} piperazine buffer, pH 10.0 and 0.6 mL Rothenfusser reagent (final pH close to 6.7) were mixed with 250 µL of raw or pasteurised milk samples or of cheese suspension in a test tube. The enzymatic reaction was started by addition of 0.6 mL of 1.0 mmol·L^{-1} hydrogen peroxide, freshly prepared. The mixture (2.45 mL final volume) was gently mixed at room temperature (22 °C) and incubated for 20 s for milk samples or 40 s for cheese suspensions. Clarifying Reagent® (2 mL) was added and the tube was vigorously shaken and incubated at 37 °C for 2 min for milk samples or 3 min for cheese suspensions. Absorbance at 560 nm of the clarified mixture was measured against a blank tube within 10 min. The blank tube contained all reagents and boiled milk instead of a milk sample.

2.2.3. Alkaline phosphatase assay

Alkaline phosphatase activity was measured by the method of Linden and Pâquet [13], modified by using the Clarifying Reagent® [14]. The hydrolysis of *p*-nitrophenylphosphate by alkaline phosphatase liberated *p*-nitrophenol, whose absorbance was measured at 420 nm. A sample solution of milk or cheese (0.5 mL) was gently mixed with 1 mL 0.15 mol·L^{-1} diethanolamine / HCl buffer, pH 10.6, containing 5 mmol·L^{-1} *p*-nitrophenylphosphate, and incubated for between 15 and 60 min at 37 °C. Clarifying Reagent® (2 mL) was then added and the tube was vigorously shaken and incubated at 37 °C for 2 min for liquid milk samples or 5 min for cheese suspension samples. Absorbance at 420 nm of the clarified mixture was measured against a blank tube within 20 min. ALP activity was expressed in µmol of *p*-nitrophenol liberated per mL of milk and per minute by using a standard curve of *p*-nitrophenol.

2.3. γ-GT kinetic analysis

In order to obtain kinetic parameters for γ-GPNA, the transpeptidation activity was measured at 37 °C for 15 min using 0.025 to 2 mmol·L^{-1} γ-GPNA as the donor substrate in 0.1 mol·L^{-1} Tris-HCl buffer, pH 8.5 with 20 mmol·L^{-1} glycylglycine as the acceptor. The Michaelis constant for glycylglycine was determined using 1.5 mmol·L^{-1} γ-GPNA and 2 to 60 mmol·L^{-1} glycylglycine. 0.02 U of commercial γ-GT was used for each experiment.

2.4. Heat treatments of milk

Heating (50 to 80 °C ± 1 °C for 15 to 4500 s) of milk samples was carried out in triplicate in a temperature-controlled oil-bath using ultra-thin glass tubes (outer diameter 6.8 mm, inner diameter 5.6 mm). Immediately after heating, the glass tubes were cooled in an ice-bath. Residual activities were calculated by subtracting appropriate blank values from the measured absorbances and expressed in percentages of the absorbances obtained from unheated controls.

The D values (time required for a 1 log reduction in activity) were determined at the tested temperatures by linear regression of plots of log residual activity versus time. The z values (increase in temperature required for 1 log change in D value) were determined by linear regression of plots of log D values versus temperature.

3. RESULTS AND DISCUSSION

3.1. Improvement of the γ-GT activity measurement method

Comparative preliminary investigations with raw, pasteurised and boiled milks had shown that 0.1 mol·L^{-1} Tris-HCl buffer used in the reference method of Patel and Wilbey [21] gave satisfying results for the clarification of mixture in the proposed method. The Clarifying Reagent® rendered the mixture transparent and increased the pH to about 12. At this pH value, γ-GT was inactive and the absorbance remained stable in time.

The absorbance of *p*-nitroaniline released by the enzymatic reaction was measured at 410 nm where γ-GPNA has only a low absorbance. The molar extinction coefficient (ε) of *p*-nitroaniline was determined to be 10 100 L·mol^{-1}·cm^{-1} at 410 nm in 0.1 mol·L^{-1} Tris-HCl buffer, pH 8.5 in the presence of 40 mmol·L^{-1} glycylglycine. This value was higher than the ε value (8 800 L·mol^{-1}·cm^{-1}) reported by Baumrucker [3] at pH 8.0. The ε value lowered to 6 300 L·mol^{-1}·cm^{-1} in the same buffer containing the Clarifying Reagent®.

In the absence of an acceptor substrate, hydrolysis and autotranspeptidation of the γ-GPNA can occur [27]. This substrate has a low affinity for the acceptor site of the γ-GT and can be used at low concentration to determine the kinetic parameters of the hydrolysis reaction [1, 29]. The D-γ-glutamyl-*p*-nitroanilide is also appropriate for studying hydrolysis but not autotranspeptidation [29]. The presence of 40 mmol·L^{-1} glycylglycine prevents the hydrolysis of γ-GPNA and the reaction of autotranspeptidation. Glycylglycine is generally chosen because it supports high catalytic activity compared with many other acceptor substrates (dipeptides and amino acids), and also because it gives the lower degree of substrate inhibition [27]. Moreover, glycylglycine, whose pK_a (8.21) is near the optimum pH of the enzyme, exhibits high solubility in the conditions of the assay.

The affinity constant for the donor substrate determined from a double reciprocal plot by using the proposed method was 0.6 mmol·L^{-1}. The K_M value for glycylglycine was 6.7 mmol·L^{-1}. Therefore, the

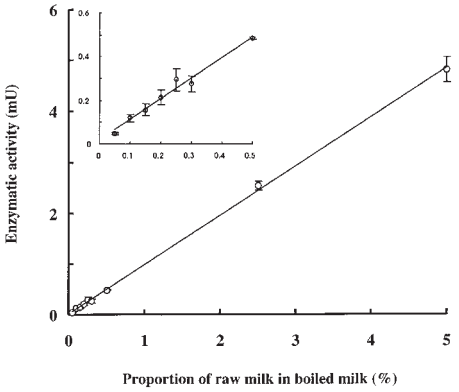

Figure 1. Activity of γ-glutamyl transpeptidase (expressed in mU) in boiled whole milk supplemented with raw milk. The insert shows the γ-glutamyl transpeptidase activities obtained with proportions of raw milk less than 0.5% ($n = 3$).

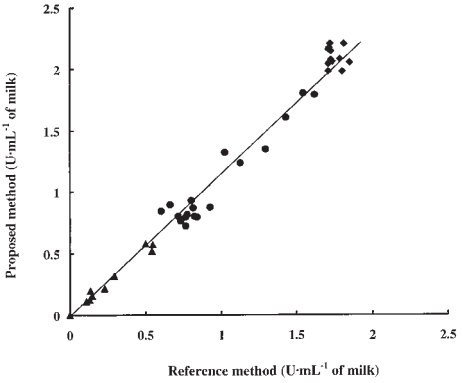

Figure 2. Correlation between the proposed method for γ-glutamyl transpeptidase activity measurement and the reference method of Patel and Wilbey [21]. Forty bovine milk samples were analysed by the two methods ($n = 5$). Results are expressed in U·mL^{-1} of milk; the regression equation was $y = 1.2\,x - 0.06$. (◆) Raw milk, (▲) pasteurised milk, (●) different dilutions of one sample of raw milk in boiled milk.

substrate concentrations used in the proposed method were about 6.5 and 6 times greater than the K_M values determined for γ-GPNA and for glycylglycine, respectively.

Using the proposed method, the reaction rate was linear at 37 °C for at least 20 min with the equivalent of 0.4 γ-GT unit/assay. A series of 20 replicates on the same pasteurised milk sample led to the determination of an enzyme activity of 0.066 ± 0.011 U·mL^{-1} of milk with satisfying repeatability (CV: 3%). Figure 1 shows that γ-GT activity was linear with increasing enzyme concentration up to the equivalent of 5 µL of raw milk in 100 µL of sample. The detection limit of γ-GT was determined as the smallest amount of raw milk added to boiled milk that produced a significant ($P \leq 0.05$) increase in response. A detection limit of 0.1 µL raw milk was determined; it corresponded to a γ-GT activity of 7.1 mU·mL^{-1}. McKellar et al. [16] report that a significant activity ($P \leq 0.05$) is detected with the addition of 0.2% of raw milk to the pasteurised milk samples; it corresponded to an activity of 13 mU·mL^{-1}.

The measurement of γ-GT activity in 40 bovine milk samples (Fig. 2) by the reference method of Patel and Wilbey [21] and by the proposed method led to the regression equation $y = 1.2\,x - 0.06$ with a good coefficient of correlation ($r = 0.98$). The activity obtained by the proposed method was slightly higher than that determined by the method of Patel and Wilbey [21] when x was superior to 0.3 U·mL^{-1}. The γ-GT activity of individual raw milk samples determined by the proposed method exhibited a weak variability with a mean value of 2.09 ± 0.07 U·mL^{-1} (minimal value 1.97 and maximal value 2.20 U·mL^{-1}; $n = 12$). The γ-GT activity of commercial pasteurised milk samples was 0.27 ± 0.20 U·mL^{-1} (minimal value 0 and maximal value 0.58 U·mL^{-1}; $n = 10$). Since assays in raw milk indicated only a weak seasonal variation [16, 21], the observed variability of γ-GT activity in pasteurised milk might reflect differences in heat load treatment.

3.2. Heat inactivation of milk γ-GT compared with ALP and LPO

Figure 3 shows the influence of various time-temperature treatments on the residual activity of milk γ-GT (determined by the proposed method), ALP and LPO. ALP was the most heat-sensitive enzyme; it retained less than 10% activity after 5 min heat treatment at 65 °C. Complete inactivation was obtained by heating at 69 °C for 15 min (Fig. 3A) which is in good

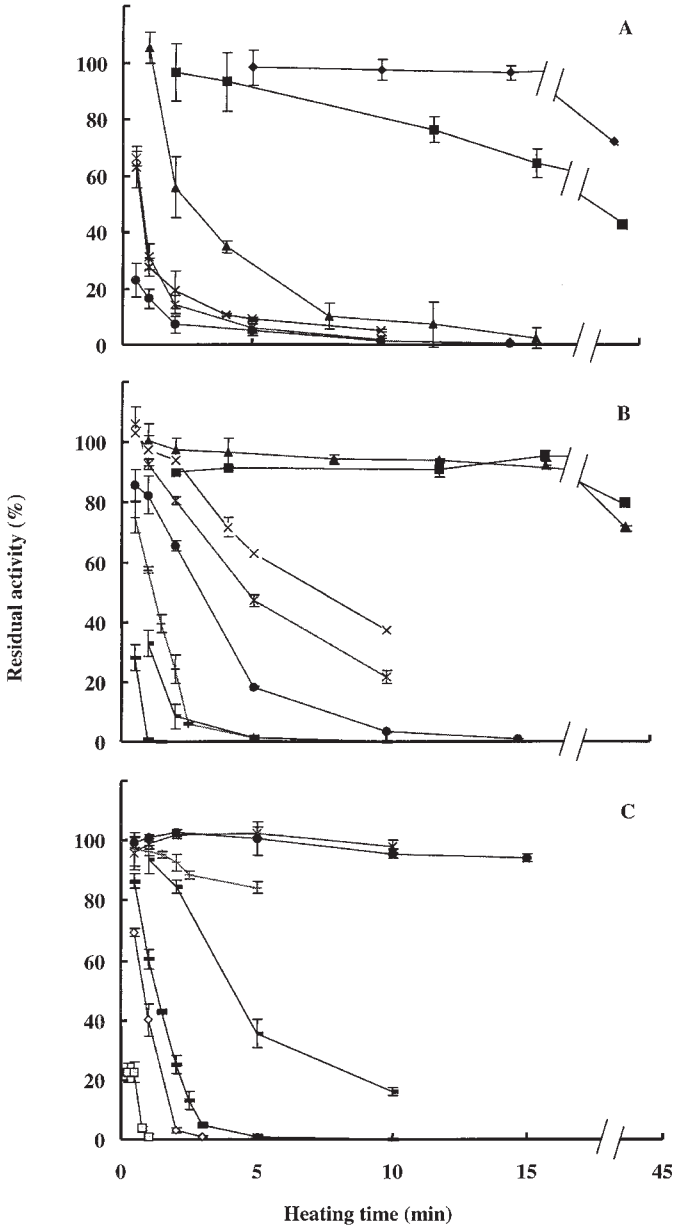

Figure 3. Residual activity of alkaline phosphatase (A), γ-glutamyl transpeptidase (B), and lactoperoxidase (C) as a function of heating time. Milk was heated at: (◆) 50 °C; (■) 55 °C; (▲) 60 °C; (×) 65 °C; (∗) 67 °C; (●) 69 °C; (+) 71 °C; (▪) 73 °C; (—) 75 °C; (◇) 77 °C; (□) 80 °C for different times (n = 9).

agreement with Dos Anjos [5] who reports a complete loss of activity at 70 °C for 10 min. γ-GT was more heat-stable than ALP: it retained nearly 50% of enzyme activity after heating at 65 °C for 5 min, and 5% of residual activity after 10 min at 69 °C. Complete inactivation was obtained after 10 min at 71 °C or 1 min at 75 °C (Fig. 3B). Dos Anjos [5] reports a complete inactivation of γ-GT after 15 min heating at 70 °C. McKellar et al. [16] and Zehetner et al. [31] obtain a complete loss in γ-GT activity after 16 s at 77 °C or 15 s at 78–80 °C without any reactivation or variation in the activity after a storage period longer than 3 d at 4 °C. LPO was the most heat-stable enzyme. Complete inactivation was obtained after 1 min of holding time at 80 °C (Fig. 3C). According to the mode of heating, the inactivation time of LPO reported in the literature varies between 5 s and 3.5 min at 80 °C [6, 7, 17].

Table I gives the times required to obtain 50% enzyme inactivation (ALP, γ-GT and LPO) at the different temperatures. At 69 °C, it was less than 0.1 min for ALP, whereas it was 24 min at 71 °C for LPO. For γ-GT, this time was 3 min at 69 °C and 1.2 min at 71 °C. No ALP activity is detected in industrial pasteurised milk, while it can retain residual activities of γ-GT and LPO [30].

Figure 4 confirms that γ-GT thermostability was between that of ALP and LPO. No activity was retained after 1 min heat treatment at 71 °C for ALP (extrapolated data), 77 °C for γ-GT and 80 °C

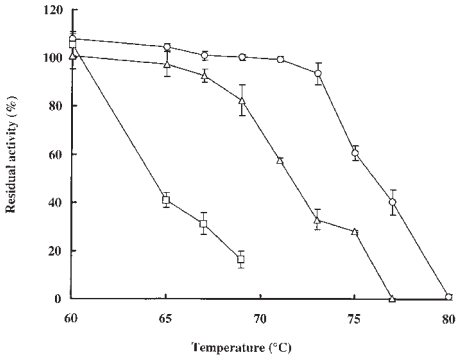

Figure 4. Residual activity of milk γ-glutamyl transpeptidase (△), alkaline phosphatase (□), and lactoperoxidase (○) after one minute of heating between 60 and 80 °C ($n = 9$).

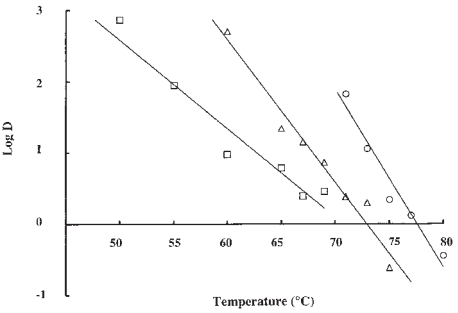

Figure 5. z-value determination for γ-glutamyl transpeptidase (△; $R^2 = 0.96$), alkaline phosphatase (□; $R^2 = 0.94$), and lactoperoxidase (○; $R^2 = 0.95$) in the range 50–80 °C. D-value: time required at each temperature to reduce enzyme activity to 10% of its original value. z-value: increase in temperature required for 1 log change in D-value ($n = 9$).

Table I. Times (min) required for 50% enzyme inactivation at different temperatures.

	Temperature (°C)									
Enzymes	55	60	65	67	69	71	73	75	77	80
γ-Glutamyl transpeptidase	nd	nd	7.5	5	3	1.2	0.4	< 0.1		
Alkaline phosphatase	25.5	2	0.8	0.7	< 0.1					
Lactoperoxidase	nd	nd	nd	nd	nd	24	4	1.5	0.9	< 0.1

nd: not determined (residual activity > 50%).

Table II. Thermal stability of γ-glutamyl transpeptidase, alkaline phosphatase and lactoperoxidase: comparison of the D-values and z-values determined in the present study with those reported in the literature.

Enzyme	Temperature (°C)	D-value (min)	z-value (°C)
γ-Glutamyl transpeptidase	67	14	5.0
	69	7.2	
Proposed method	71	2.4	
	73	1.7	
	75	0.34	
McKellar [15]	71	19.8	4.97
	74	4.14	
	77	1.73	
Zehetner et al. [31]	-	-	4.8
Andrews et al. [2]	71	4	5.4*
	72	1.83	
	75	0.66	
Alkaline phosphatase	50	725	8.0
	55	88	
Present study	60	9.5	
	65	6.1	
	67	2.5	
	69	2.9	
Griffiths [7]	62	20	4.9
	64	8	
	66	3	
	70	0.5	
	69	0.25	5.1
Schlimme et al. [25]	63	8.3	8.0
Lactoperoxidase	73	11.4	4.1
	75	2.2	
Present study	77	1.3	
	80	0.35	
Olszewski and Reuter [19]	-	-	3.7
			9.4
			5.9
Griffiths [7]	65	26.6	5.4
	70	15.7	
	75	0.8	
	80	0.08	

D-value: time required for 1 log reduction in activity at a specific temperature;
z-value: increase in temperature required for 1 log change in D-value;
(*): calculated from D-values given by Andrews et al. [2].

for LPO. The reactivation of ALP, that occurs in pasteurised milk stored at a temperature higher than 20 °C, or during melting of butter to obtain serum for analysis, has led McKellar et al. [16] to propose γ-GT assay as an alternative method for the control of milk pasteurisation. Furthermore, γ-GT is more abundant in milk than ALP, its concentration being about 700% higher [5]. Thus, γ-GT residual activity could allow better estimation of heat load than ALP and LPO activities.

Curves used for the determination of z-values (Fig. 5) also show that γ-GT had intermediate heat sensitivity between ALP and LPO. However, the calculated z-value for γ-GT was close to that for LPO (Tab. II). Both D- and z-values determined in the present work for γ-GT and LPO were in the range of previously reported values [7, 15, 19, 31]. The z-value of 8 °C determined in this work for ALP is similar to the value found by Schlimme et al. [25], but is greater than the other values of Griffiths [7] (Tab. II). The observed differences in D- and z-values probably resulted from the different heating conditions, the time required to reach the heating temperature not being the same with a plate heat-exchanger and with a water- or oil-bath.

3.3. Residual γ-GT activity in cheese compared with that of ALP and LPO

The label "made with raw milk" on the packaging requires that products are obtained without any previous heat treatment of milk, including thermisation. Residual ALP and γ-GT enzymatic activities present in soft cheeses (Camembert) manufactured with either raw or pasteurised milk are shown in Table IV. No LPO activity was recovered in any of the samples tested, probably because a major part of the enzyme was left in whey during cheese processing, and the residual amount of LPO remaining in the cheese was certainly below the detection limit of the method. γ-GT and ALP, which are membrane-bound enzymes, remain in cheese after the curd draining.

The range of values of specific γ-GT activity in Camembert cheese made with raw milk was 8.55 to 11.68 $U \cdot g^{-1}$ of dry matter

Table III. γ-Glutamyl transpeptidase and alkaline phosphatase activities in Camembert cheese samples made with raw or pasteurised milk ($n = 5$).

Cheese	γ-Glutamyl transpeptidase ($U \cdot g^{-1}$ of dry matter)	Alkaline phosphatase ($U \cdot g^{-1}$ of dry matter)
Raw milk		
Camembert 1	9.73 ± 0.10	0.41 ± 0.00
Camembert 2	8.65 ± 0.13	0.40 ± 0.00
Camembert 3	10.20 ± 0.21	2.03 ± 0.01
Camembert 4	8.55 ± 0.06	1.47 ± 0.01
Camembert 5	11.68 ± 0.20	1.80 ± 0.01
Camembert 6	11.58 ± 0.18	-
Mean value	10.06 ± 1.37	1.22 ± 0.77
Pasteurised milk		
Camembert 7	0.06 ± 0.00	0.13 ± 0.00
Camembert 8	1.55 ± 0.02	0.18 ± 0.00
Camembert 9	0.00	0.29 ± 0.01
Camembert 10	1.10 ± 0.05	0.02 ± 0.01
Mean value	0.67 ± 0.77	0.15 ± 0.11

(Tab. III). Those values were about 14 times greater than those determined for Camembert cheese made with pasteurised milk, which ranged between 0.00 and 1.55 $U \cdot g^{-1}$ of dry matter. ALP specific activity was in the range 0.40 to 1.80 $U \cdot g^{-1}$ of dry matter in Camembert cheese made with raw milk, while it was 0.02 to 0.29 $U \cdot g^{-1}$ of dry matter for cheese made with pasteurised milk (Tab. III). The positive response to the ALP test is the most accepted parameter for the characterisation of a raw-milk cheese [23]. ALP activities were low and the difference between raw and pasteurised milk cheese was not significant, which made it difficult to differentiate them. On the contrary, the identification of raw and pasteurised milk cheese was not confused by using γ-GT.

4. CONCLUSION

The proposed method for the quantification of γ-GT activity in raw and pasteurised milk samples was well correlated with another method using the same donor and acceptor substrates. Our colorimetric method was very easy, and more sensitive and rapid without requiring a spectrophotometer with a thermostated cuvette. This method allowed the measurement of the γ-GT activity of 10 samples in the same series of assays, which is not the case with the reference method. It could be applied for routine characterisation of mild heat treatment of milk. The proposed method could be used to measure γ-GT activity in Camembert cheese, which could be of interest in controlling the heat load of cheese milk. This method, which is technically applicable to Emmental cheese (data not shown), will be used for studying γ-GT residual activity in a controlled sampling of hard cheese.

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