Changes in N-Acetyl-B-D-Glucosaminidase and B-Glucuronidase Activities in Milk during Bovine Mastitis

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ABSTRACT

To determine the N-acetyl-B-D-glucosaminidase (NAGase) and B-glucuronidase (B-Gase) activities in mastitic milk, basic enzyme assay conditions, distribution of NAGase and B-Gase, comparison of their activities with California Mastitis Test scores, and the effects of the milking process on their enzyme activities were examined. The mean NAGase and B-Gase activities in milk macrophages were about threefold higher than those of milk and blood polymorphonuclear cells. Very little NAGase activity appeared to be associated with blood mononuclear cells, whereas a relatively higher B-Gase activity was observed. California Mastitis Test scores of each group (1 to 5) appeared to be well correlated (r = 0.86 for NAGase and 0.92 for B-Gase) with the levels of NAGase and B-Gase activity. The milking process was least effective in the normal milk, but some variations of enzyme activities during milking in mastitic milk were found. Changes in NAGase and B-Gase activities in quarter milk were well monitored during the course of clinical mastitis.

Key words: N-acetyl-B-D-glucosaminidase, B-glucuronidase, bovine mastitis, marker enzyme, mastitis monitoring.

RÉSUMÉ

Afin de déterminer l'activité de la N-acétyl-B-D-glucosaminidase et de la B-glucuronidase, dans le lait mammiteux, les auteurs évaluèrent les conditions basales des épreuves enzymatiques, le distribution des deux enzymes précités, leur activité par rapport aux résultats du test de la mammite de Californie et l'influence de la traite sur l'activité des dits enzymes. Leur activité se révéla environ trois fois plus grande dans les macrophages du lait que dans ses neutrophiles et ceux du sang. L'activité du premier enzyme s'avéra faible, dans les mononucléaires du sang, contrairement à celle du deuxième. Les résultats des groupes #1 à #5 du test de la mammite de Californie affichèrent une bonne corrélation avec le degré d'activité des enzymes expérimentaux (r = 0,86 pour le premier et 0,92 pour le deuxième). La traite de vaches saines n'exerça que très peu d'influence sur le degré d'activité des deux enzymes précités, contrairement à ce qui se produisit chez celles qui donnaient du lait mammiteux. Les variations de l'activité des deux enzymes, dans le lait de chaque quartier, firent l'objet d'une surveillance étroite, durant la phase clinique de la mammite.

Mots clés : N-acétyl-B-D-glucosaminidase, B-glucuronidase, mammite bovine, enzyme marqueur, monitorage de la mammite.

INTRODUCTION

Mastitis is the most common and economically important disease of dairy cattle. Information on the prevalence and incidence of clinical and subclinical mastitis is a significant aid in providing the mastitis status of the herd, cow or quarter. The most common method used for diagnosis of subclinical mastitis is the bacteriological examination in conjunction with a direct or indirect cell count. Other mastitis diagnostic tests on quarters are based on the changes in conductivity (1,2), serum albumin (3) and antitrypsin (4,5). A high-capacity, easily performed and inexpensive monitoring system will enable the veterinarian and research workers to maintain a constant record of mastitis in the herd. Kitchen *et al* (6-9) reported a series of studies concerning the N-acetyl-B-D-glucosaminidase (EC 3.2.1.30) (NAGase) in bovine milk as a marker enzyme for bovine mastitis and applied it to determine the extent of mammary gland epithelial cell damage. The assay conditions for the determination of the NAGase described by Kitchen were designed to monitor changes in cell counts between the levels of 2.5×10^5 and 2×10^6 cells/mL. These levels are useful for screening chronic mastitis and for the estimation of bulk milk somatic cell levels. In our preliminary study, we applied the NAGase assay system as described by Kitchen *et al* (8) to assay milk from severe clinical mastitis cases, and a lack of linearity of the enzyme catalyzed reaction was found.

Kitchen (6) did not use the B-glucuronidase (EC 3.2.1.31) (B-Gase) assay procedure because of its long incubation periods and its lower activity. Nevertheless, the B-Gase seems to be a useful marker enzyme (13).

This study was performed to determine the basic enzymological assay conditions using the above mentioned enzymes for monitoring the health of the bovine udder. In addition, the distribution of the enzymes and comparison of their activity with the results of California Mastitis Test (CMT) scores was investigated. The effect of the milking process on enzyme activities was studied and the results were applied for monitoring the changes in the enzyme levels during the course of clinical mastitis.

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MATERIALS AND METHODS

ANIMALS

Sixty-two Holstein Friesian cows were used: The 54 cows were raised in Rakuno Gakuen University grounds and in a commercial dairy farm. They were used in obtaining quarter milk samples for enzyme assays. Eight cows, with a naturally occurring acute clinical mastitis and maintained in four local dairy farms were used in providing milk for monitoring the enzyme activities of infected quarters during the course of bovine mastitis. In this study, the criteria of clinical mastitis was based on typical systemic signs, the nature of the secretions obtained from the infected quarters, the degree of udder swelling, bacteriological findings and CMT tests. These findings were all positive, and the animals were treated with fluid and bacterial therapy.

COLLECTION OF MILK SAMPLES

The first few squirts of milk were discarded and 2 mL samples of quarter foremilk were collected under aseptic condition in sterilized plastic tubes during the routine herd surveys. For the comparison of enzyme activities with the California Mastitis Test (CMT; P-L tester, Zenyaku Co, Ltd, Japan), CMT was performed and the CMT scores were recorded from CMT 1 to 5. To examine the effect of milking process on NAGase and B-Gase activities, five quarter milk (A-E) from five mastitic cows (CMT; 2-5) and quarter milk from a cow (CMT; negative) were collected continuously into 50 mL plastic tubes, and then 5 mL of each collected sample was transferred into 10 mL tubes and used for enzyme assay. Samples were transported to the laboratory and stored at -30° C until used.

ISOLATION OF LYMPHOCYTES AND NEUTROPHILS FROM BLOOD

Lymphocytes were isolated from bovine peripheral blood using Ficoll-Conray density centrifugation as described previously (10). Isolated lymphocytes were washed twice with phosphate buffered saline (PBS, pH 7.4). The cell concentration was then adjusted to 2×10^6/mL.

Neutrophils were isolated from red blood cell layer obtained by density centrifugation, the red blood cell layer was treated with cold 0.2% NaCl solutions for 20 seconds with gentle shaking and then equal volumes of cold 1.6% NaCl solutions were added to the cells (twice). The remaining cells were washed twice with PBS by centrifugation at 200 g for 5 min and the cells were suspended in PBS at a concentration of 2×10^6 polymorphonuclear cells/mL. The cell suspensions were centrifuged at 500 g for 10 min and the supernatants were discarded. Equal volumes of 0.1% triton X-100 were added to the cell pellets and solubilized thoroughly. The resultant solutions were used for enzymic assays.

ISOLATION OF MACROPHAGES AND NEUTROPHILS FROM MAMMARY SECRETIONS

Aliquots (50 mL) of sterilized physiological saline were infused via the teat canal into quarters of an early dry period (-5 days after drying off) cow, milked immediately. The mammary secretions were filtered through a cotton gauze and centrifuged at 1000 g for 10 min. The cream layer and skim milk was discarded and aliquots of PBS were added and centrifuged at 150 g for 15 min. Cells were adjusted to a concentration of 5×10^6 cells in PBS containing 10% bovine serum. The mononuclear cells (MNCs) and neutrophils were then separated by Ficoll-Conray density centrifugation, as described above. Mammary macrophages were isolated from MNCs by the procedure described previously (11). Cells adhering to the culture dishes (Falcon 3002, Becton Dickinson, California) were referred to as macrophage-rich fractions. Neutrophils were isolated from the cells at the bottom of tubes according to the manner described above. Resultant cells were referred to as neutrophil-rich fractions.

ENZYME ASSAY

NAGase: NAGase was essentially measured as described by Kitchen *et al* (7,8) by a fluorometric procedure using 4-methylumbelliferyl-N-acetyl-B-D-glucosaminide (Sigma Chemicals Co, Missouri) as substrate. The enzymic reaction was started by adding 30 μL of a milk sample to a tube containing 200 μL of 2 mM 4-methylumbelliferyl-N-acetyl-B-D-glucosaminide in 0.25 M citrate buffer (pH 4.4), and incubated for 5 min at 37° C.

B-Gase: B-Gase activity was measured by a fluorometric procedure using 4-methylumbelliferyl-N-acetyl-B-D-glucuronide (Nakarai Chemicals, Ltd., Kyoto, Japan) as substrate. The enzyme reaction was started by adding 50 μL of a milk sample to a tube containing 200 μL of 2.5 mM 4-methylumbelliferyl-B-D-glucuronide in 0.25 M citrate buffer (pH 5.2), and incubated for 10 min at 37° C. The enzyme volume, the incubation time and substrate concentration were varied in order to optimize the assay conditions. The reaction was terminated by the condition of 5.5 mL of 0.1 M carbonate buffer (pH 10) and the released 4-methylumbellliferone was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm, using a spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan). Standard curve ranges for 4-methylumbelliferone were from 1 to 8 μM for NAGase and from 0.1 to 0.7 μM for B-Gase. The enzyme activity was expressed as nanomoles of liberated 4-methylumbelliferone per minute in 1 mL of milk (nM/min/mL).

STATISTICAL ANALYSIS

Data were analyzed using the Student's *t* test.

RESULTS

ASSAY CONDITIONS

To optimize the enzyme assay conditions, effects of enzyme concentrations, pH and substrate concentrations on NAGase and B-Gase activity were determined (Figs. 1 and 2). The NAGase derived from severe clinical mastitic milk catalyzed reaction was linear with time for an incubation of 10 min when enzyme concentrations of 30 μL were used. In the case of normal milk and enzyme concentrations of 30 μL and 50 μL, the reaction was linear up to 20 min. The B-Gase derived from severe clinical mastitic milk catalyzed reaction was linear with time of incubation for at least 20 min using

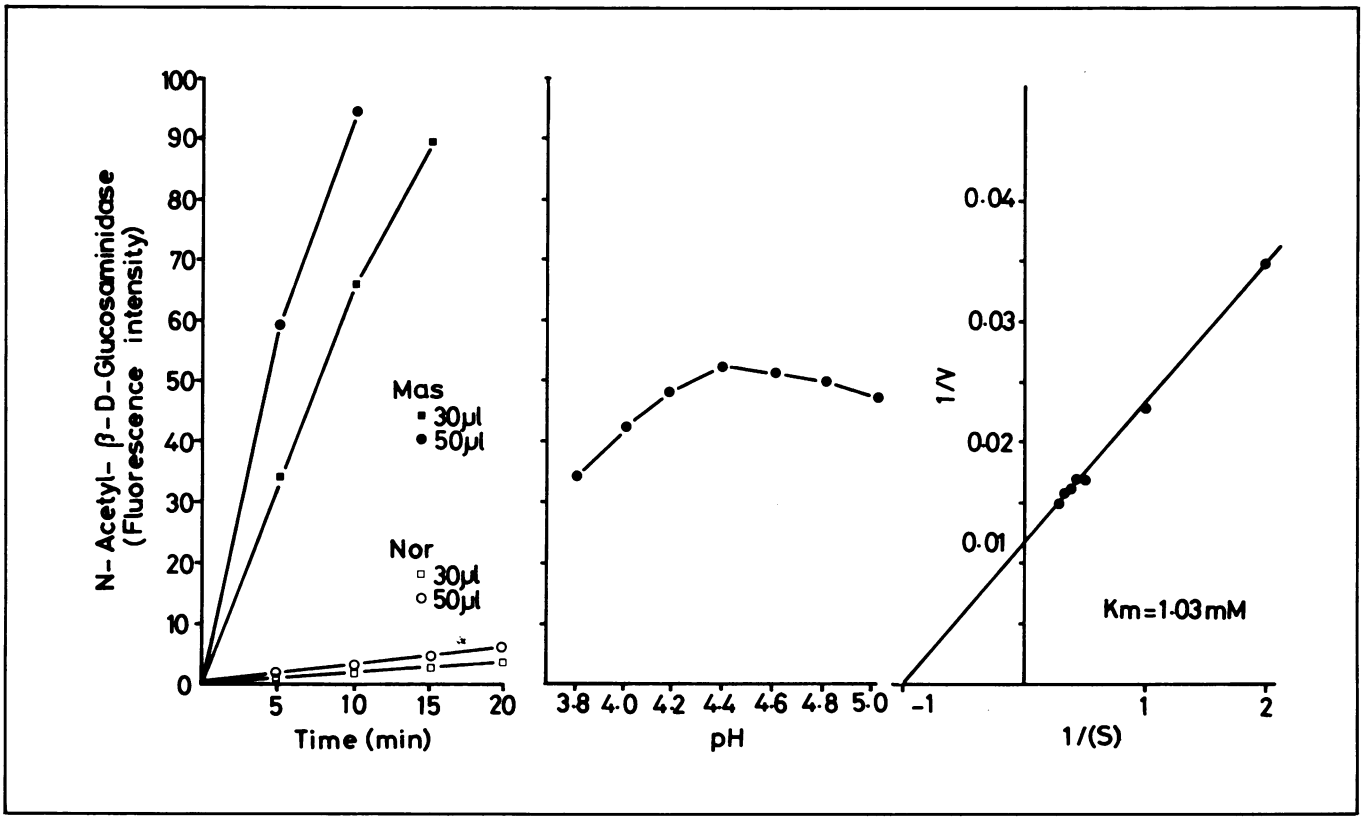

Fig. 1. Effects of enzyme concentrations, pH and substrate concentrations on N-acetyl-B-D-glucosaminidase activity. Mas: mastitic milk, Nor: normal milk.

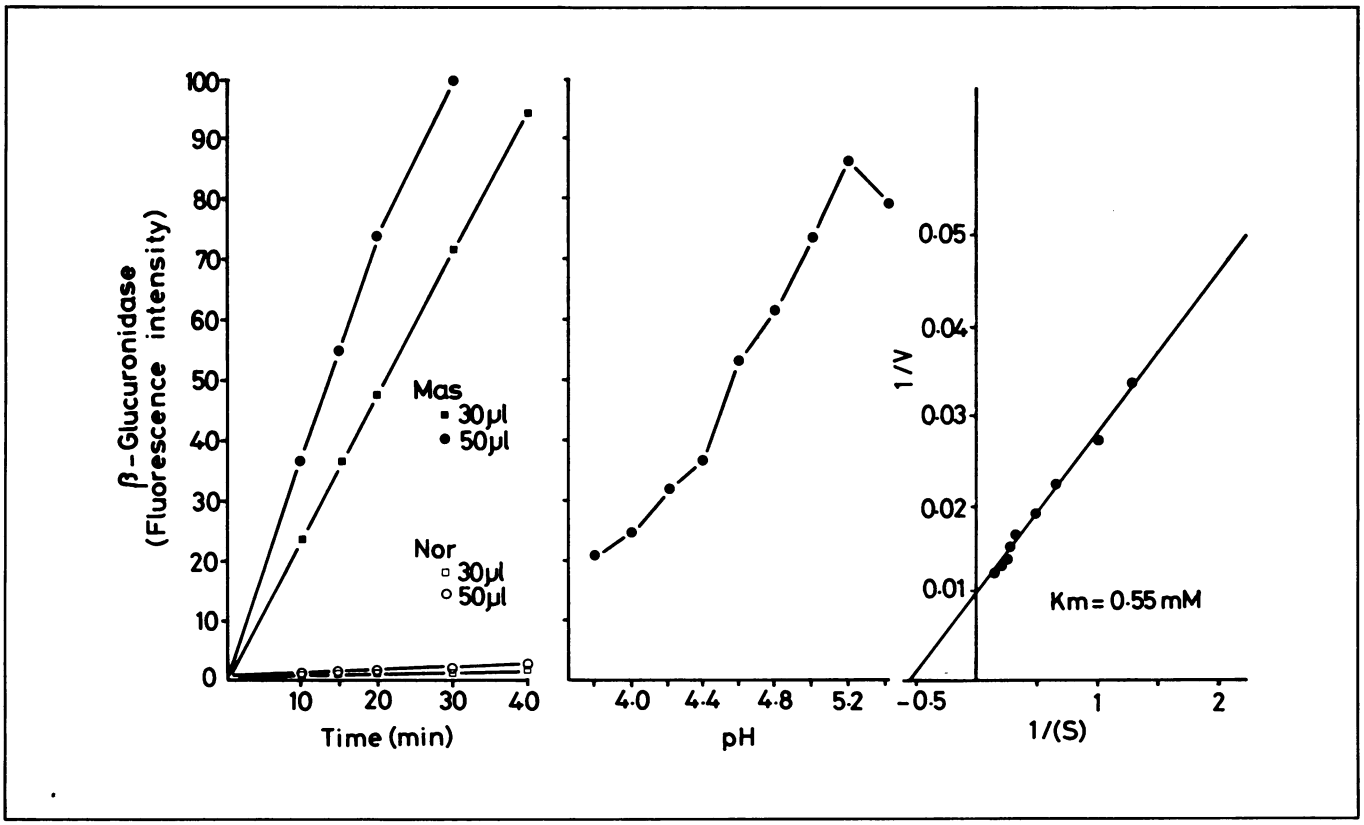

Fig. 2. Effects of enzyme concentrations, pH and substrate concentrations on B-glucuronidase activity. Mas: mastitic milk, Nor: normal milk.

50 μL enzyme concentrations, and linearity of the reaction increased to 40 min when enzyme concentrations of 30 μL were used. For the case of normal milk and enzyme concentrations of 30 and 50 μL, the rate of enzymic reactions was very slow. Optimal pH for NAGase and B-Gase assay were 4.4 for NAGase and 5.2 for B-Gase under citrate buffer. The effects of substrate concentration on the rate of hydrolysis of 4-methylumbelliferyl-N-acetyl-B-D-glucosaminide and 4-methylumbelliferyl-B-D-glucuronide are shown in Figures 1 and 2 in the form of a Lineweaver Burk Plot. The Km values for NAGase and B-Gase in mastitic milk were 1.03 and 0.55 mM, respectively.

DISTRIBUTION OF NAGase AND B-Gase ACTIVITY

At the cellular level, mean NAGase and B-Gase activities in milk macrophages were about threefold higher than those of milk and blood polymorphonuclear cells (Table I). Very little NAGase activity appeared to be associated with blood mononuclear cells, whereas relatively higher B-Gase activity was observed. In blood serum, higher NAGase activity was observed, while B-Gase was considerably lower in activity as compared with milk macrophages.

RELATIONSHIP BETWEEN ACTIVITIES OF NAGase AND B-Gase AND CMT SCORES

The CMT results of sample milk were compared with their NAGase and B-Gase activities (Table II.) The CMT scores of each group appeared to be well correlated ($r = 0.86$ for NAGase and $r = 0.92$ for B-Gase) with the levels of NAGase and B-Gase activity. Although wide ranges of enzyme activities were found within each class of milk, mean enzyme activity values of each group appeared to be representative. Apparently, above CMT score 3, their activities increased remarkably. Mean NAGase and B-Gase activities of CMT score 5 showed increments of 33-fold for NAGase and tenfold for B-Gase, in comparison with those of normal milk.

EFFECTS OF MILKING PROCESS ON NAGase AND B-Gase ACTIVITIES

NAGase and B-Gase activities were continuously monitored from fore milk to stripping milk (Fig. 3). For NAGase activity below 25 nM/min/mL and B-Gase activity below 0.3 nM/min/mL, the changes due to the effect of milking process was less variable, while some samples with NAGase activity above 50 nM/min/mL and B-Gase activity above 1 nM/min/mL had wide changes due to the milking process. In general, enzyme activities in stripping milk were slightly higher than those of fore milk.

NAGase AND B-Gase ACTIVITY LEVELS DURING THE COURSE OF MASTITIS

NAGase and B-Gase activities in each quarter fore milk were monitored in eight cases of clinical mastitis with natural infection (Figs. 4 and 5). For A, the highest NAGase activity was determined on the first day. After three days, the activity decreased rapidly but decreased moderately after seven days. For B and C, enzyme activity values were remarkably higher in the first three days, but thereafter decreased rapidly and maintained at almost normal levels. Although the enzyme activities of D, E, F and G decreased consistently, they still retained the "mastitic levels" from 7 to 21 days. The general changes of B-Gase activity was similar to that of NAGase, though lower activities of NAGase were observed in D and E as compared with others. Rather higher activities of B-Gase were found, still unrestored to the normal level.

TABLE I. Distribution of N-Acetyl-B-D-Glucosaminidase and B-Glucuronidase Activity

Source	(n)	N-Acetyl-B-D-Glucosaminidase[a] mean±SD	N-Acetyl-B-D-Glucosaminidase[a] range	B-Glucuronidase[a] mean±SD	B-Glucuronidase[a] range
Blood serum	10	48.99 ± 21.33	14.94 - 84.88	0.29 ± 0.07	0.23 - 0.41
Blood polymorphonuclear cells[b]	9	8.85 ± 3.15	6.11 - 14.27	0.22 ± 0.05	0.16 - 0.32
Blood lymphocytes[b]	11	2.03 ± 0.86 [c]	0.68 - 3.39	0.19 ± 0.06 [c]	0.09 - 0.26
Milk polymorphonuclear cells[b]	5	6.66 ± 1.21 [c]	5.45 - 8.15	0.16 ± 0.01 [c]	0.15 - 0.19
Milk macrophages[b]	5	25.00 ± 8.91	15.63 - 33.29	0.62 ± 0.24	0.36 - 0.98

[a]Activity: nanomoles of 4-methylumbelliferone produced in 1 min/mL of milk

[b]2×10^6 cells/mL

[c]Significant difference $P < 0.05$

TABLE II. Comparison between Activities of N-Acetyl-B-D-Glucosaminidase, B-Glucuronidase and CMT Scores

CMT Score	(n)	N-Acetyl-B-D-Glucosaminidase[a] mean±SD	N-Acetyl-B-D-Glucosaminidase[a] range	B-Glucuronidase[a] mean±SD	B-Glucuronidase[a] range
1	55	5.73 ± 3.82	1.91 - 14.33	0.27 ± 0.17	0.16 - 0.86
2	44	15.28 ± 10.51	4.78 - 40.11	0.40 ± 0.19	0.20 - 0.92
3	14	27.70 ± 20.10	6.21 - 67.81	0.55 ± 0.33	0.23 - 0.98
4	13	62.86 ± 20.73	25.15 - 90.73	1.56 ± 0.98	0.73 - 3.81
5	37	180.06 ± 92.12	47.80 - 464.83	2.76 ± 2.59	0.79 - 11.37

[a]Activity: nanomoles of 4-methylumbelliferone produced in 1 min/mL of milk

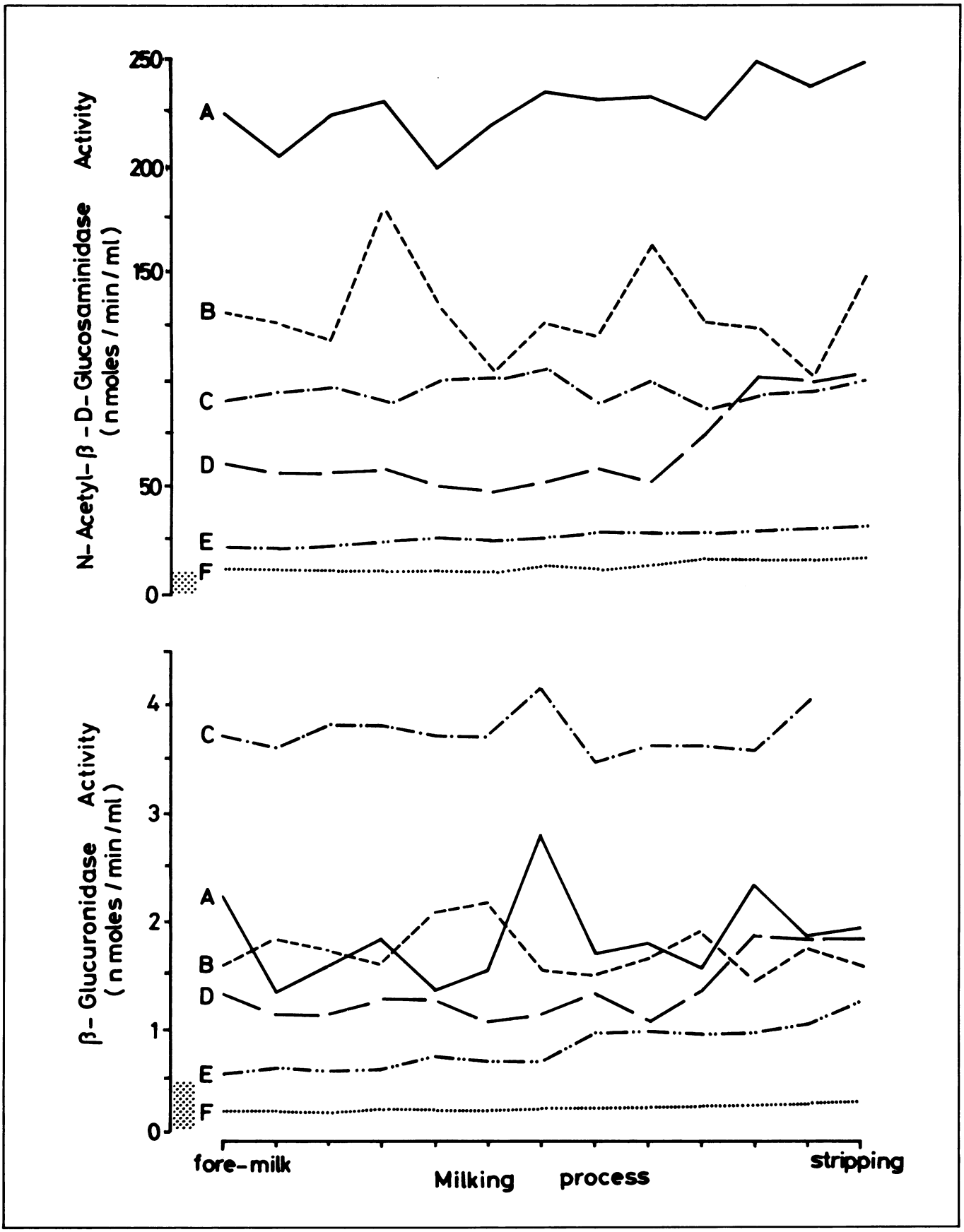

Fig. 3. Changes in N-acetyl-B-D-glucosaminidase and B-glucuronidase activities during milking process (fore-milk to stripping milk). A-F represent the quarter milk samples. The shaded area represents the range of the normal milk.

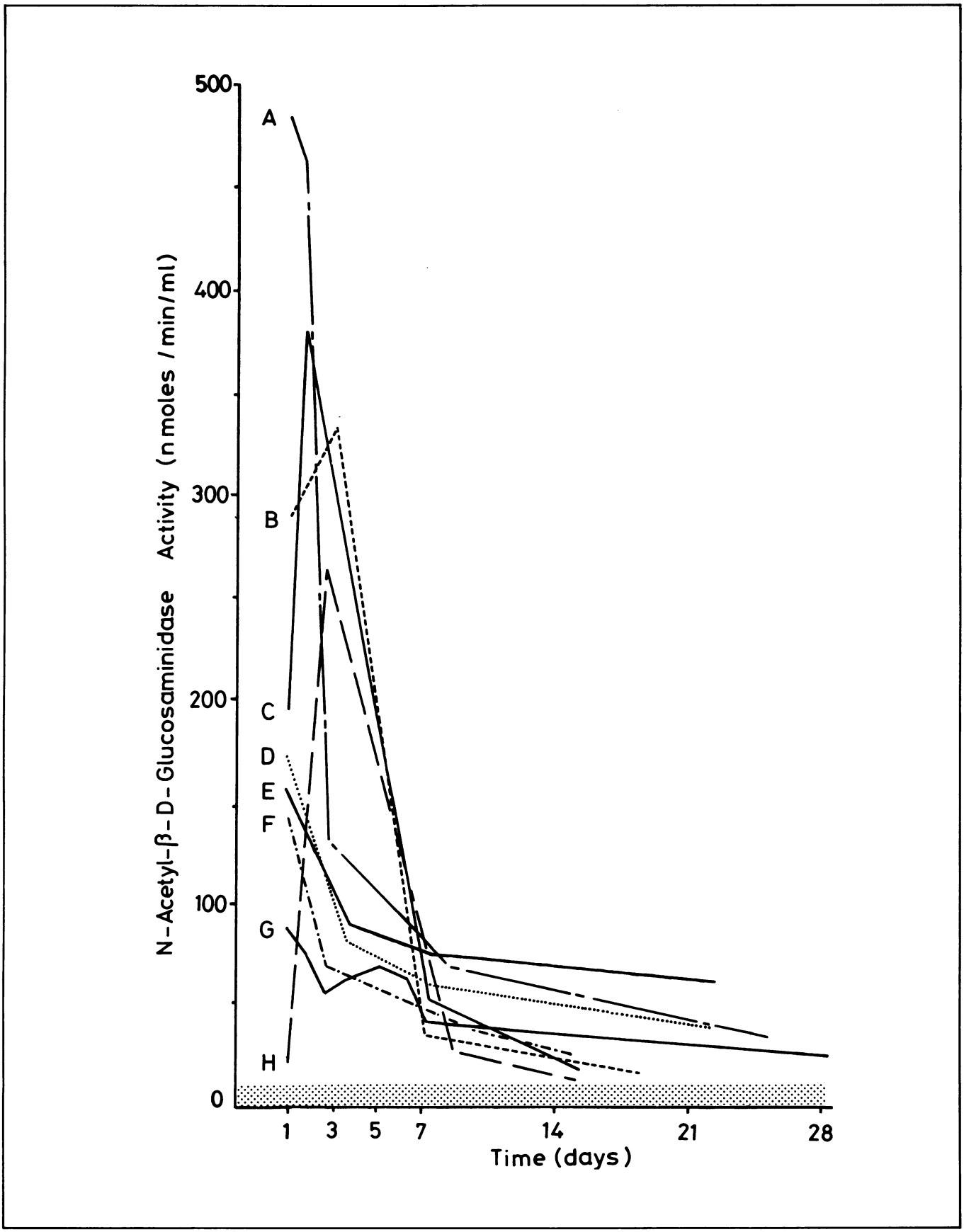

Fig. 4. Changes in N-acetyl-B-D-glucosaminidase activity during clinical mastitis. A-H represent the quarter milk levels from eight cows with clinical mastitis. The shaded area represents the range of the normal milk.

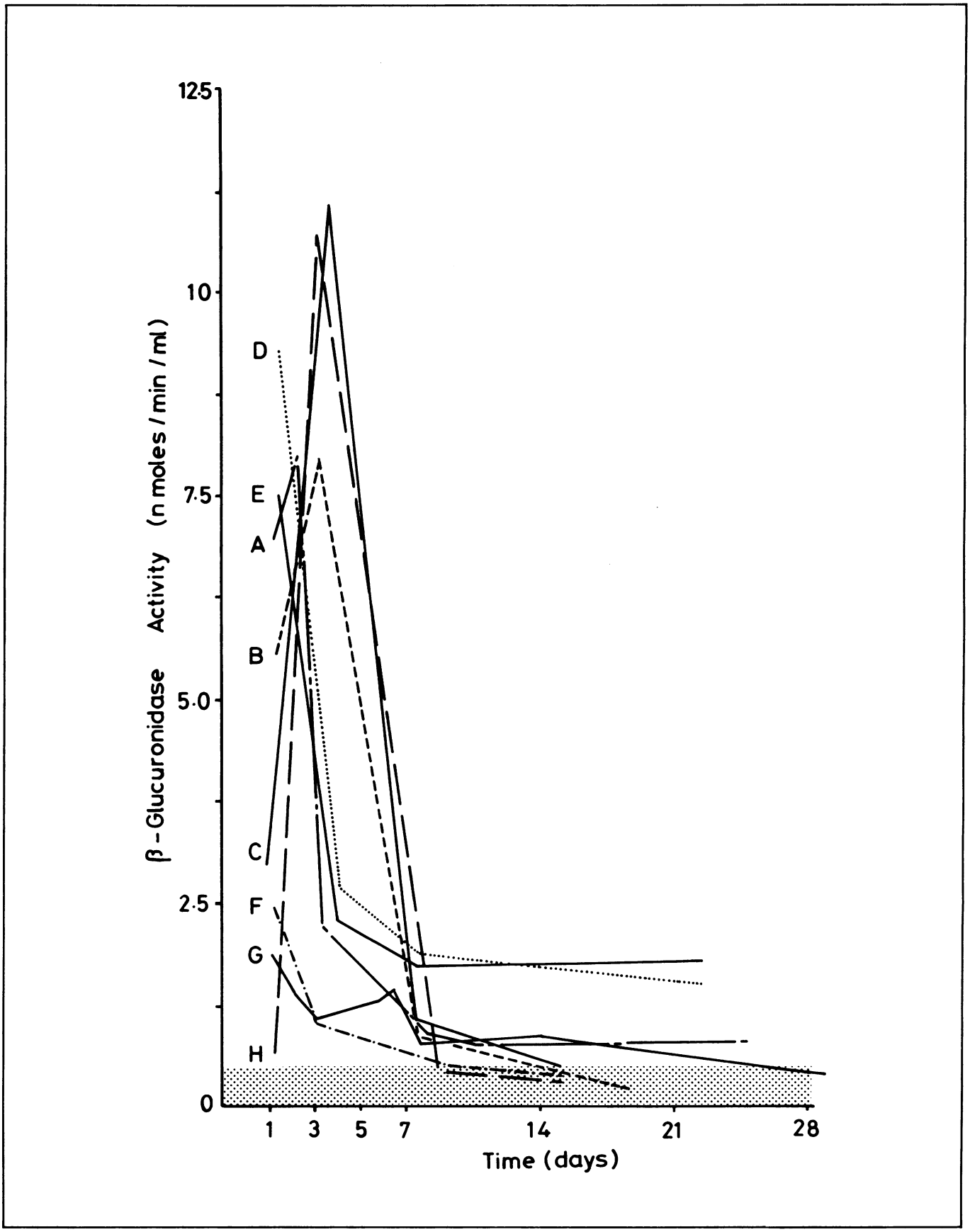

Fig. 5 Changes in B-glucuronidase activity during clinical mastitis. A-H represent the quarter milk levels from eight cows with clincial mastitis. The shaded area represents the range of the normal milk.

DISCUSSION

Mellor (12) reported firstly, the presence of NAGase in bovine milk and suggested that bovine milk NAGase is derived wholly from the leukocytes and its level in milk may be a convenient measure of mammary gland infection. Kiermeier and Güll (13) reported previously that B-Gase activity in cow milk was measured by a colorimetric method using phenolphthalein B-glucuronide as substrate, and indicated that if B-Gase was derived from leukocytes, its activity in milk was a useful measure for bovine mastitis. Kitchen *et al* (6-9) applied NAGase assay for estimation of somatic cell counts, as a marker for mastitic milk and for monitoring bulk milk. Obara *et al* (14,15) reported on the relationship between milk compositional changes and their enzyme activities. Ulberth *et al* (16) reported that in detecting mastitis, sensitivity and specificity of the NAGase activity assay obviously did not differ from the cell count determination. NAGase in bovine milk seems to be a useful marker enzyme for detection of abnormal udder secretions. In the present study, application of enzyme assays to determine the levels of the two enzymes in severe clinical mastitis was attempted in order to obtain information concerning an inflamed mammary gland.

Assay conditions for NAGase and B-Gase activities in milk of severe clinical mastitic cases were measured (Figs. 1 and 2). In order to obtain a linearity of enzymatic reactions, enzyme volumes and incubation time were 30 μL and 5 min respectively for NAGase. The optimal pH and apparent Km values were similar to those reported previously by Mellor (12). The optimal conditions for B-Gase assay were: using 2.5 mM of substrate concentration in citrate buffer, 50 μL of milk, and incubation of 10 min. The fluorometric assay of B-Gase was very simple, highly reproducible and less time consuming (10 min) than the colorimetric assay.

Differences in lysosomal enzyme activities between classes of animal leukocytes (five species) have been reported by Healy (17). In cattle, a higher NAGase activity was found in granulocytes. Kitchen *et al* (8) reported that mammary gland secretory cells contained high levels of NAGase and appeared to be the major source of the enzyme in milk whereas NAGase was from other sources (white blood cells and blood serum) and contributed only a minor proportion (5-15%) of the total activity in milk. A higher activity of NAGase than of B-Gase was observed in serum, macrophages and neutrophils. From these results, we assumed that in mastitic milk, most of the NAGase and B-Gase activity may be derived from blood serum, neutrophils and macrophages associated with inflammatory process, as well as mammary secretory cells.

Our results showed that NAGase and B-Gase activities in normal quarter milk were 9.5 nM/min/mL and 0.45 nM/min/mL, respectively. These enzyme activities are used as thresholds for the classification of milk.

Changes in NAGase and B-Gase activity during naturally occurring clinical mastitis was monitored. The activities in acute clinical mastitis increased remarkably from 25 to 500 nM/min/mL for NAGase and from 2.5 to 10 nM/min/mL for B-Gase. After the first week of adequate antibacterial therapy and treatments, these levels decreased gradually. In chronic mastitis, these changes were small. Interesting findings were observed. A dairy farmer suspected mastitis in case H and collected the milk. The NAGase and B-Gase activities were within the normal range on the first day but their enzyme levels increased rapidly three days after the suspected mastitis. Thereafter, the levels decreased drastically following the antibacterial therapy. Such a case of NAGase and B-Gase activity patterns demonstrate "scissors" form of acute clinical mastitis. In quarters D, E, F and G, the onset of inflammation may have passed, and thereafter, levels decreased during the monitoring process. This study demonstrated the importance of NAGase and B-Gase for the detection of mastitis. The assays confirmed and monitored the course of clinical mastitis and stressed the application of NAGase and B-Gase assay for the future follow-up of clinical mastitis in cattle.

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