Diagnosis of Bovine Mastitis by Determination of Lysosomal Enzyme Activity in Milk

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Mastitis is one of the most important diseases of dairy cows. To detect the disease in the field, California Mastitis Test (CMT) has been used extensively. In this test, the number of leukocytes appearing in milk during inflamation is estimated semiquantitatively. At the same time, changes of pH of the sample are determined by changes of color of the indicator.

Kitchen[9] reported in a review on methods for diagnosis of bovine mastitis that the milk components available for diagnosis of bovine mastitis were cell counts, lactose, Na, K, and Cl level, and the level of some types of milk serum protein and enzyme. Paying attention to the lysosomal enzyme in milk,[8,9] he studied the significance of estimation of this enzyme in relation to the cell count in milk, and concluded that the estimation of N-acetyl-β-D-glucosaminidase (NAGase) was the most useful for diagnosis of mastitis.

Recent studies have shown the possibility of diagnosing mastitis by the estimation of electric conductivity of milk. This method has an advantage that the electric conductivity is measured continuously during milking. Some workers reported that the electric conductivity of foremilk is different from that of stripping.[1,2,3]

In the present investigation, firstly, we tried to clarify the relationship between the lysosomal enzyme level and the cell count or milk components including lactose, protein and chloride,[13,14] and pointed out that NAGase was the most reliable lysosomal enzyme in milk for diagnosis of mastitis.[14] Secondly, we examined changes of NAGase in milk with the progress of mastitis induced experimentally in goats. Thirdly, we studied changes of enzyme activity, electric conductivity, cell count and organisms throughout the milking process. The suitability of NAGase activity as a diagnostic test for bovine mastitis was discussed.

Relationship between milk components and lysosomal enzymatic activity in abnormal milk

Samples of foremilk were collected separately at morning milking. They were subjected to the modified California Mastitis Test (PL tester). Lysosomal enzymatic activities such as β-glucuronidase, NAGase, acid phosphatase, α-mannosidase and arylsulphatase were estimated as soon as possible after sampling by the method reported by Kitchen.[8] The cell count in milk was made by the direct microscopic method.[7] Fat, protein, and lac-

Table 1. Correlation coefficient between cell count and each enzyme activity in whole milk or milk ingredients

	Cell count	Log cell count
NAGase	0.67***	0.76***
β-glucuronidase	0.59***	0.72***
Acid phosphatase	0.68***	0.43***
α-mannosidase	0.10	0.32**
Arylsulphatase	0.31**	0.40***
Cl	0.55***	0.62***
Lactose	−0.56***	−0.53***
Protein	0.17	0.45***
Fat	0.08	0.37**
SNF	−0.20	0.02

Significance levels; ** $P<0.01$, *** $P<0.001$.

Table 2. Correlation coefficient among enzyme activities and components in whole milk or milk ingredients

	β-glucu-ronidase	Acid phosphatase	α-man-nosidase	Aryl-sulphatase	Cl	Lactose	Protein	Fat
NAGase	0.66***	0.62***	0.27*	0.67***	0.87***	−0.81***	0.36**	0.40***
β-glucuronidase		0.31**	0.46***	0.43***	0.47***	−0.34**	0.65***	0.56***
Acid phosphatase			0.06	0.47***	0.53***	−0.67***	0.15	0.25*
α-mannosidase				0.28*	0.21	−0.19	0.45***	0.48***
Aryl-sulphatase					0.76***	−0.68***	0.35**	0.45***
Cl						−0.86***	0.15	0.31**
Lactose							−0.07	−0.30*
Protein								0.74***

Significance levels; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

tose were determined by an automatic infrared milk analyzer (ILMA), and chloride by the method of Schales and Schales.[12]

Table 1 shows the relationship between the cell count and each enzymatic activity and component of milk. Lysosomal enzymes, NAGase, β-glucuronidase, and acid phosphatase exhibited high correlations with cell count. When the cell count was expressed in logarithm, NAGase showed a correlation coefficient ranging from 0.68 to 0.77 and β-glucuronidase from 0.59 to 0.72. The chloride and lactose level showed correlation coefficients of 0.62 and −0.55, respectively, with the logarithmic cell count.

Table 2 indicates correlation coefficients among the lysosomal enzymes and components of milk in the present investigation. NAGase showed considerably high correlations with β-glucuronidase, acid phosphatase and arylsulphatase. Chloride exhibited remarkably high correlations with NAGase and arylsulphatase and a low correlation with β-glucuronidase. Lactose correlated to NAGase, acid phosphatase, and arylsulphatase with $r=-0.68$ to -0.81. Milk protein showed considerably high correlations with β-glucuronidase and α-mannosidase.

From these results, it was concluded that NAGase can be used as an indicator of inflammation in udders and that β-glucuronidase might also be utilizable.

Diagnosis of bovine mastitis by means of NAGase activity in milk

1) Estimation of NAGase activity

The method of measuring NAGase activity in whole milk is shown in Fig. 1. Milk samples were diluted 2.5 to 11 times by addition of citrate buffer solution (200 mM, pH 4.5). To 0.5 m*l* of this test material, 1 m*l* of 0.33 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide solution (in citrate buffer solution) was added as the substrate. The resulting mixture was incubated at 37°C for 1 hr and 3 m*l* of 1 M glycine (pH 10.5) was added to stop the reaction at the end of the incubation. After adding chloroform (3.5 m*l*), the mixture was shaken, and then centrifuged at 3,000 rpm for 30 min. The amount of p-nitrophenol liberated was determined by colorimetry at 410 nm of the supernatant layer. The NAGase activity was expressed as the amount of p-nitrophenol produced per 1 m*l* of milk for 1 min.

2) Relationships between NAGase activity and somatic cell count, lactose, chloride and lactoferrin

At morning milking, foremilk samples were collected separately from each quarter of 16 holstein cows in the field. Some of the cows had mastitis. NAGase activity, cell count in whole milk (by direct microscopic method[7]), and concentrations of lactose (by infrared

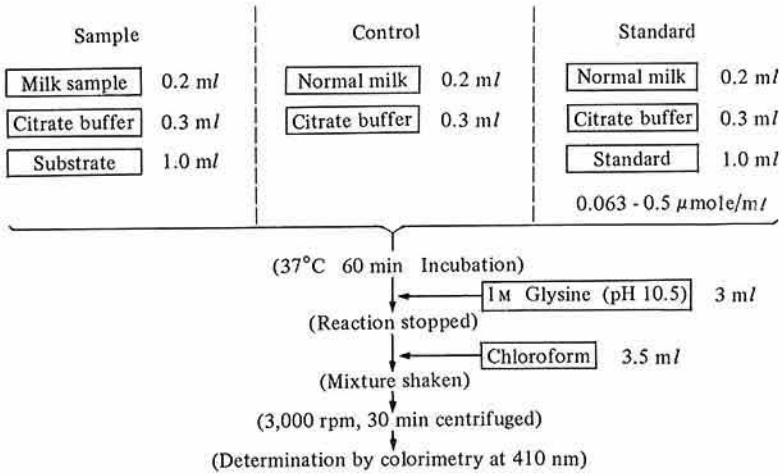

Fig. 1. Method of determination of N-acetyl-β-D-glucosaminidase activity in milk

automatic analyzer), chloride (by Schales and Schales' method[12]), and lactoferrin (by single radial immno-diffusion method[11]) were measured.

Correlation coefficient between NAGase activity and each of cell count, lactose, and chloride was 0.72, −0.80, and 0.88, respectively. Lactoferrin is a whey protein that increases remarkably in concentration in the inflammatory stage of mastitis.[5,6] It originates from secretory cells of mammary glands.[4] Such a high correlation coefficient as 0.91 was obtained between NAGase activity and lactoferrin.

NAGase activity in the skim milk fraction and in the soluble whey fraction was 92.1% and 82.6%, respectively, of the activity in the whole milk. Kitchen et al.[10] reported that the supernatant fraction of mammary gland homogenates contained 90% of the total NAGase activity in the whole mammary gland. From these results, it appears that cells of the mammary gland are the main source of NAGase secretion in milk.

In healthy udders of cow, only a small amount of NAGase is secreted from the cytosol of secretory cells. When inflammation occurs, secretory cells are injured and variable amount of NAGase may be released into milk depending on the intensity of inflammatory changes. The amount of NAGase activity in milk probably indicates the severity of inflammation of udders, i.e. degree of injury of mammry glandular tissues. Thereby NAGase activity of milk provides a useful indicator of the udder damage for the diagnosis of mastitis.

NAGase activity of milk and mammary glands of goats with experimentally-induced mastitis

Five goats in lactation were used in this experiment. Right udder of each goat was infused with 10^8 CFU of *Staph. aureus* (Kitami 3-9D strain) to induce an inflammatory response. Milk and blood samples were collected at morning milking for 30 days after the infusion to determine NAGase and β-glucuronidase activities and cell count in milk. The similar sampling was carried out from noninfused control udders on the same days. At the end of the experiment, the mammry glands were sampled from the sacrificed goats, and after washing, their pieces were homogenized in cold (5°C) water with a mixer. The homogenate was filtered through two

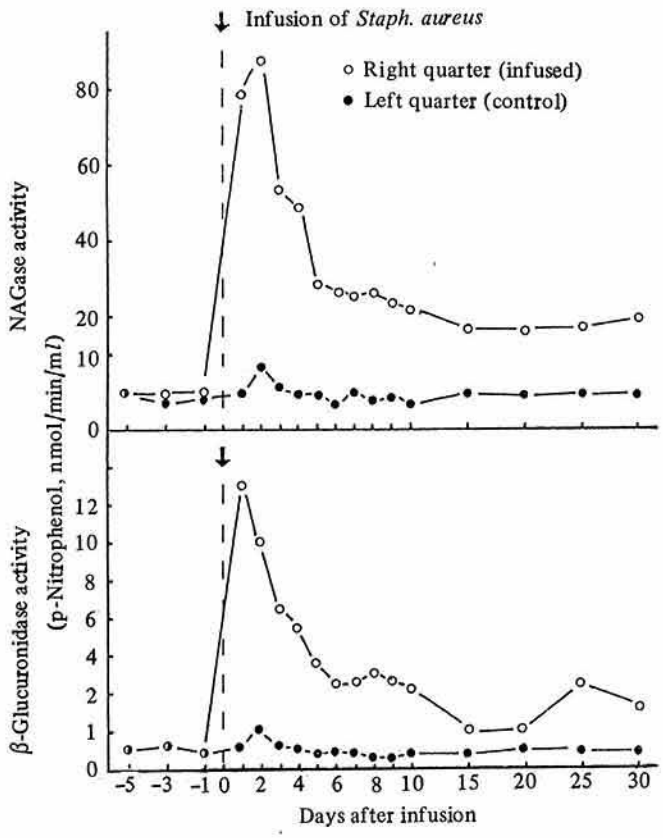

Fig. 2. Changes of enzyme activities of milk sampled from udders with or without experimentally induced mastitis in goats

layers of gauze, centrifuged at 50,000×g for 60 min (5°C), and clear supernatant was collected for determinations of NAGase, β-glucuronidase and acid phosphatase activities.

Fig. 2 shows changes in mean enzyme activity of milk from the infected udders and noninfected (control) udders of the 5 goats during the experimental period. NAGase activities in the milk from the treated quarter increased to a maximum level of 80 nmole/min/ml on 2 to 3 days after infusion, followed by a decrease to 20 nmole/min/ml on about 6th day, and the value which was about 4 times that of the control udders was maintained until the end of the experiment. In the control udders, a slight sympathetic response in enzyme activity was observed on the 2nd day. Changes of β-glucuronidase activity in milk after infusion showed the same tendency as that of NAGase activity.

Table 3 shows NAGase, β-glucuronidase and acid phosphatase activities in the infected and noninfected udders of the 5 goats. The mean values of NAGase and β-glucuronidase activities in infected udders were significantly higher than those in control udders. There was no significant difference in acid phosphatase activities between the infected and noninfected udders. Considerable differences in NAGase and β-glucuronidase activities observed among udders of each goat were dependent on the intensity of inflammatory changes observed histo-pathologically, in infected udders.

From these results, the determination of NAGase activity seems to be the best method that can replace monitoring udder epithelial cell damage.

Table 3. NAGase, β-glucuronidase, and acid phosphatase activities in infected and noninfected (control) udders of 5 goats

Goat No.	NAGase		β-glucuronidase		Acid phosphatase	
	Control	Infected	Control	Infected	Control	Infected
1	495	644	25.4	29.7	492	401
2	292	407	26.9	27.3	688	725
3	349	511	23.3	33.1	407	640
4	313	734	13.8	44.0	263	600
5	381	483	19.8	47.0	382	339
Average±SD	366±71	556±12	21.8±4.7	36.2±7.9	446±141	542±147
	*		*		N.S	

Significance levels: * $P<0.05$
Unit: nmole/min/g

Diagnosis of bovine mastitis by means of changes of some enzyme activities in milk during the milking process

Changes in NAGase and β-glucuronidase activities, electric conductivity, cell count, and organisms in milk during the milking process were examined with 14 quarters of 7 holstein cows of various ages and at various stages of lactation. Besides, foremilk and stripping milk samples were collected from two quarters at several times during milking through a

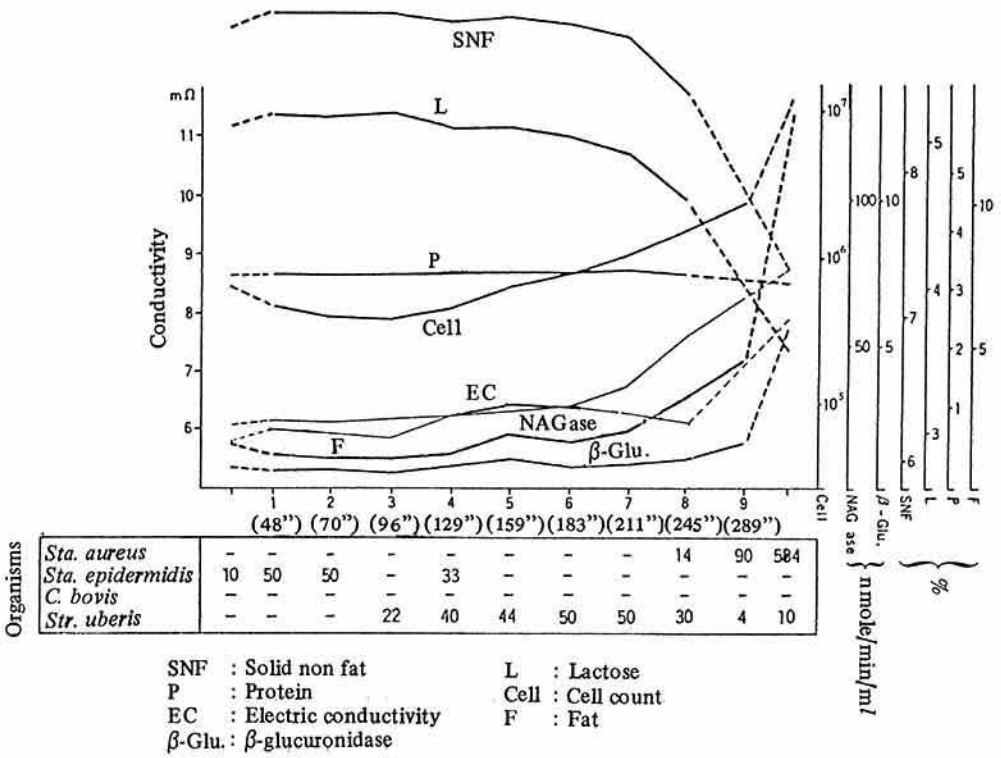

Fig. 3. Changes of enzyme activity, cell count, electric conductivity, organisms and milk components in mastitis-quarter milk during milking progress

T-pipe connected to a milker. Milk conductivity was recorded continuously throughout the milking process. Samples of foremilk, middle milking and strippings were taken to determine the electric conductivity, CMT and cell count by using the TOA electrical conductivity meter (CM-50AT), PL tester (ZENYAKU) and Colter counter (ZB-1), respectively. NAGase and β-glucuronidase were determined by p-nitrophenol method. Milk fat, milk protein and lactose were also determined by MILKOSCAN (infrared absorption of milk).

Fig. 3 shows changes of enzyme activities, cell count, electric conductivity, organisms and milk components in mastitis quarter milk during milking. NAGase activity increased gradually with the progress of milking from 15 nmole/min/m*l* in foremilk to 120 nmole/min/m*l* in strippings. Somatic cell count, electric conductivity and β-glucuronidase activity showed the same patterns during milking.

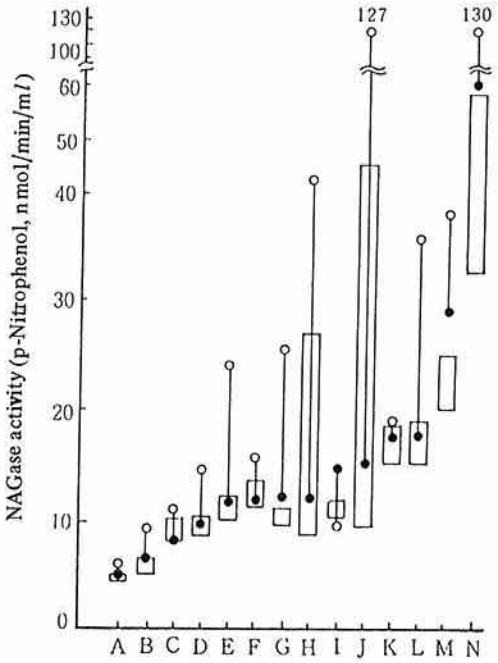

Fig. 4–1. Changes of NAGase activity in all quarters

Concentration of lactose and SNF decreased in the process of milking. This quarter was infected with *Str. uberis*, *Staph. aureus*, and *Staph. epidemids*. *Staph. aureus* was isolated only from the milk at the final stage of milking and from strippings. Normal quarter milk which had stable low value in NAGase activities showed low value in electric conductivity, and cell count as well, without containing any organisms.

NAGase activity of milk in the process of milking of all quarters of cows used in this experiment is shown in Fig. 4–1, in which the quarters were arranged in order from minimum (A) to maximum (N) activity in foremilk. Normal quarters with NAGase activity in milk lower than 10 nmole/min/m*l* showed a little change in the enzyme activity during milking. However wider variation in the enzyme activity during milking was observed in quarters which had the enzyme activity higher than 10 nmole/min/m*l*.

Fig. 4–2 shows patterns of change of NAGase activity in six quarters sampled during milking. The patterns were divided

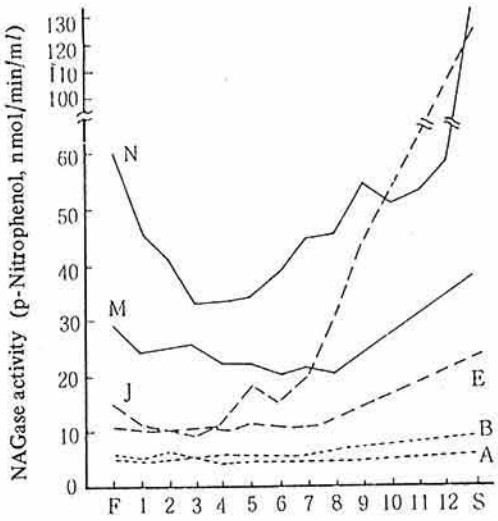

Fig. 4–2. Patterns of change of NAGase activity with milking progress in six quarters

into 3 groups. Group 1: NAGase activity in milk was lower than 10 nmole/min/ml without variations during milking (Normal quarters, A and B). Group 2: NAGase activity in milk higher than 10 nmole/min/ml increased gradually and showed remarkable increase at the final stage of milking (Quarter J). Group 3: NAGase activity higher than 30 nmole/min/ml decreased until middle of milking stage and then increased again at the final stage of milking (Quarter M, N).

Results obtained from the present study indicated that: 1) Sampling for the diagnosis of mastitis should be modified to collect not only foremilk samples but also stripping samples. 2) Determination of NAGase activity and monitoring of electric conductivity during the process of milking from respective quarters are useful for the diagnosis of mastitis at an early stage of the disease.

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(Received for publication, February 22, 1984)