Arab Journal of Scientific Research, 18 (3), pp 184-188 (2000)

ORIGINAL PAPER

A Choukri

Improvement in Subclinical Mastitic Milk Detection Methods

Abstract: Subclinical mastitic milk presents a composition sensitively different from normal milk which could be reflected in milk products. Among the diagnostic methods of subclinical mastitic milk proposed, the enzymatic method (NAG-ase determination) and somatic cell count (SCC) are the most utilized. According to several authors, NAG-ase determination could be considered as one the best to detect mastitic milk.

We present here a method for the determination of NAG-ase (N-Acetyl-B-D-glucosaminidase) activity in cow's milk. The analysis procedure was derived from that of Kitchen and Middleton (1976), where the precipitation, centrifugation and filtration steps were replaced by the transparization technique. A dissolving reagent was used at the end of the enzymatic reaction which rendered the medium transparent and thus could be directly read by spectrometer.

The reproducibility of the procedure was satisfactory and good correlations with a fluorimetric enzymatic method and somatic cell count were observed. Our results show that it is possible to classify milk according to its degree of infection while being based only on the activity of NAG-ase.

Key words :Mastitis, NAG-ase, SCC, Milk, Transparization

طريقة محسنة للكشف عن التهاب الضرع تحت السريري في الحليب

علـي شكـــري

المستخلص: يعتبر مرض التهاب الضرع الذي يصيب العديد من الحيوانات الحلوبة، من المشاكل المهمة التي تواجه منتجي الألبان. و في حالة الإصابة بهذا الداء بشكل سريري أو تحت السريري، يلاحظ تغير معتبر في التركيب الكيميائي للحليب و بالتالي تنخفض قيمته التكنولوجية، وتصعب معالجته أو تحويلـه. لـذلك أصبـح من الضـروري الكشف المـبكر عن الإصـابـة بالمرض، و الذي يمكنه أن يجري عن طريق اختبار الحليب. توجد هناك طرق عديدة لإجراء الإختبار نذكر منها اختبار كاليفورنيا (CMT)، القياس التفلوري، قياس التوصيلية الكهربائية، تقدير ادينوزين ثلاثي الفوسفات (ATP) و كذا عدد الخلايا الجسمية . (SCC)

و يتفق كثير من الباحثين على أن تحديد فعالية أنزيم NAG-ase داخل الحليب من أحسن الطرق المستعملة للكشف عن الإصابة المبكرة بالمرض في الدور تحت السريري.

نقترح في إطار هذا البحث طريقة لتحديد نشاط NAG-ase في حليب البقر. المنهج التحليلي مشتق من طريقة (Kitchen & Middleton , 1976) لكنه يختلف تماماً في المراحل الأخيرة بحيث تم حذف كل من عمليات الترسيب و النـبذ المركزي و الترشيـح و اسـتبدالـهـا بـعـمـلـيـة الـتشفيف (transparization) مباشرة بعد التفاعل الإنزيمي و الحصول على وسط شفاف يسهل قياسه بواسطة المقياس الطيفي.

إختبار الطريقة المقترحة و دراسة الارتباط بين ما اقترحناه و كل من طريقة القياس التفلوري و عدد الخلايا الجسمية قد بينت نتائج حسنة . كما أنه تم تقسيم الألبان إلى ثلاثة أقسام حسب درجة الإلتهاب.

الكلمات المفتاحية

التهاب الضرع، NAG-ase، الحليب طريقة الكشف، .SCC,التشفيف

Introduction

The biological variation of N-Acetyl-B-D-glucosaminidase (NAG-ase, EC; 3.2.1.30) has permitted the diagnosis of numerous pathogenic states in men as well in animals. In milk, the level of NAG-ase varies considerably during mammary infections, which makes it a good indicator of mastitis and mastitic milk (Kitchen and Middleton, 1976; Kitchen and Al 1978; Kitchen, 1981; Nagahata *et al,* 1987). Among the technological consequences of mastitic milk, we can state the depressive effect on milk production, thermal instability and organoleptic quality alteration of various products (Serieys, 1987; Kerjean *et al*, 1986; Chassagne *et al*, 1998).

Several authors had compared NAG-ase activity with other subclinical diagnostic methods, such as antitrypsin, albumin, somatic cell count (SCC) and bacteriological analysis, and found NAG-ase level determination to be the best method for the detection of mastitis (Mattila *et al,* 1986; Riipinen, 1988; Pyrola, 1989). According to Emanuelsson *et*

A Choukri
Agro Pastoralism Institute
University Centre of Djelfa
P O Box 3117, Djelfa
Algeria

al, (1987), and Rupp and Boichard 1999), NAG-ase activity determination and leukocyte count present a similar diagnosis. The cytological technologies are numerous and diversifying, among which direct microscopic cellular numeration is considered as the reference method, while indirect tests such as the California Mastitis Test (CMT) and the Wisconsin Mastitis test (WMT) are used frequently.

The enzymatic activity of NAG-ase is measured by a quantitative method using a spectrophotometer (Kitchen and Middleton, 1976; Choukri 1990; Choukri 1997) and a fluorimetric method (Kitchen *et al,* 1978; Obara, 1985). In the present article, we propose a determination method of NAG-ase activity in cow's milk. The analysis procedure was derived from the method of Kitchen and Middleton (1976), but the precipitation, centrifugation and filtration steps were replaced by the transparization technique at the end of the enzymatic reaction.

Materials and Methods

Reagents

Commercial N-Acetyl-B-D-glucosaminidase in lyophilised form (20 mU NAG-ase from ox's kidney/ml; Boheringer Mannheim); 4 –Methylumbelliferyl-N-Acetyl-B-D glucosaminide; p-nitrophenyl –N-Acetyl- B-D-glucosaminide; 4-methylumbelliferone, sodium salt (Sigma).

The dissolving reagent (Prolabo Society) consists of a basic detergent mix and a solvent which has the clarification properties toward certain biological fluid medium. It was conducted after completion of the enzymatic reaction (Guingamp *et al,* 1989; Choukri, 1991).

Milk samples:

In thé procedure optimization, mixed whole fresh bovine milk was used. Individual whole fresh bovine milk was used in the correlation studies.

Spectrometric determination of NAG-ase activity:

The method principle is based on the hydrolysis of p-nitrophenyl N-Acetyl- B-D-glucosaminide by NAG-ase. The paranitrophenol liberated was colored yellow. After the addition of a solution to stop the enzymatic reaction, the dissolving reagent was added to obtain a transparent liquid. Color intensity was read directly with a spectrometer at 410 nm. The spectrometric determinations were read off a calibration curve prepared with paranitrophenol.

Results are expressed in nanomoles of released paranitrophenol per ml of the sample and time unit.

Determination of NAG-ase activity by a fluorimetric method:

The method used was proposed by Kitchen *et al,* (1978). To 0.05 ml of milk, 0.2 ml of 4–Methylumbelliferyl-N-Acetyl-B-D glucosaminidase in 0.25M citrate buffer, pH 4.6, was added. After incubation at 37°C for 15 min, the reaction was stopped by adding 5.5 ml of 0.1 M, pH 10, sodium carbonate buffer solution. Fluorescence intensity liberated from 4–methylumbelliferone was measured with a spectrofluorimeter (Shimadzu R.F 5000).

Excitation wavelength was fixed at 365 nm and emission wavelength at 450 nm, while the bandwith was fixed at excitation and emission of 5 nm. Instrument standardization was done using a solution of 4–methylumbelliferone 5 mM in 0.1 M, pH 10, carbonate buffer.

Determination of somatic cell count (SCC):

The somatic cell count was determined by fluorescence on fossomatic material (Foss-Electric, Paris).

Results and Discussion

Optimization of experimental conditions

We have optimized the experimental conditions of buffer and pH which were suitable to the enzyme's activity in the reaction medium and to the clarification at the end of the reaction. The medium system was kept stable as long as possible to allow the reading of a greater sample series.

The reaction medium is similar to that proposed by Kitchen and Middleton (1976). The optimum condition for commercial NAG-ase activity was determined in milk and in buffer medium. In the two cases the optimum pH was found to be 4.6, which was comparable to that reported by Kitchen and Middleton (1976) and Nagahata (1987), who found a value of 4.4. NAG-ase is more active at 50°C, we found, like Kitchen *et al,* (1978), that at 37°C the enzyme lost almost half of its activity.

In our conditions, the rate of paranitrophenol liberation was practically linear during 15 min and the Km constant evaluated to 1.016 mM.

Mellors (1968) reported a value of 1 mM. The same result was found by Kitchen *et al,* (1978) using the fluorimetric method, but they found Km value of 0.8 mM using colorimetric method. Finally,

Nagahata *et al*, (1987) reported a Km of 1.03 mM. Substrate stability in transparent medium had permitted us to increase its concentration to get a value of 5 times the Km.

Proposed procedure for the determination of NAG-ase activity in milk:

Based on the result obtained, we propose the following procedure. To 0.2 ml of milk, add 0.3 ml of 0.33 M, pH 4.6, citrate buffer containing 5 mM p-nitrophenyl-N-Acetyl-B-D-glucosaminide. After vortexing, incubate 15 min at 50°C. At the end of the reaction, add 1.5 ml of inactiving solution (1 vol Ethylen-Diamine tetra-acetic-acid (EDTA) 2 % pH10 + 2 vol 1M glycine–NaOH buffer, pH 10) and 1.5 ml of dissolving reagent. Follow by agitation.

The final pH is around 9.42 . The absorbency is read spectrometrically at wavelenth 410 nm. The reagents used resulted in a good clarification and the medium is stable for 30 min.

Reproducibility of the method:

The procedure proposed was satisfactorily reproducible. The same analysis repeated 33 times using the same milk gave a coefficient of variation of 3%.

Correlation with the fluorimetric method:

A good correlation was found with the fluorimetric method. The coefficient of correlation was 0.96 with 52 different samples. The correlation line is given in Figure 1.

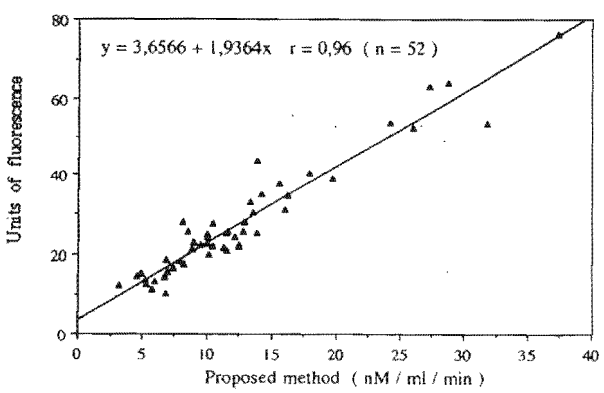

Fig. 1. Linear correlation between proposed method and fluorimetric method

Correlation with somatic cell count:

The present procedure correlated well with the number of somatic cells. The coefficient of correlation was 0.90 with 52 different samples (Figure 2). Other works show that the count of milk somatic cells correlated well with the spectrometrically measured NAG-ase activity with a correlation of 0.84 (Kitchen *et al*, 1980).

By measuring the level of NAG-ase by the semi-quantitative method using lovibond comparator, Kitchen and Middleton (1976) found a correlation of 0.87 with the somatic cell count.

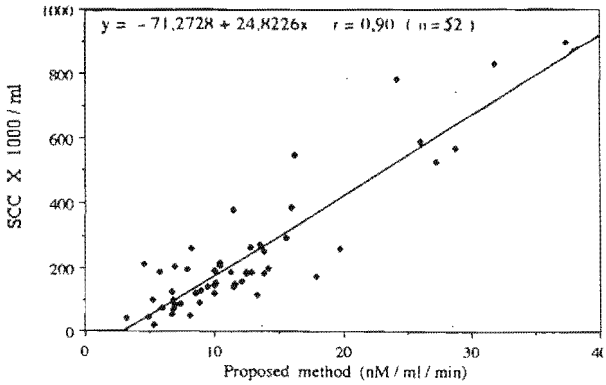

Fig. 2. Linear correlation between proposed method and SCC

Correlation between SCC and the fluorimetric methods is evaluated to 0.85 (Figure 3). The same result (0.86) was also found by Kitchen *et al*, (1978) and Kitchen (1978).

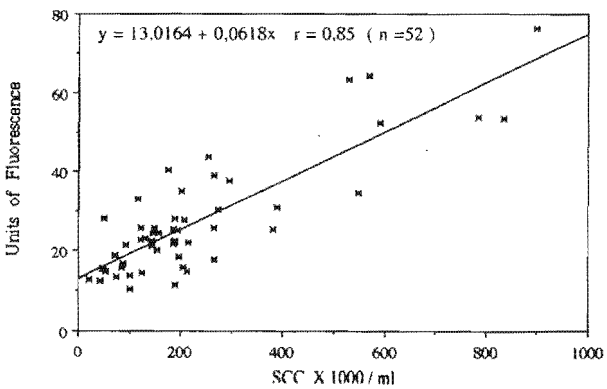

Fig. 3. Linear correlation between fluorimetric method and SCC

Milk classification following the degree of infection:

We distributed our milk samples in 3 classes according to the number of total somatic cells (SCC x 1000/ml of milk):

SCC < 200,000/ml
200,000/ml < SCC < 400,000/ml
SCC > 400,000/ml

In practice, several authors fix the value limit at 400,000 /ml. When the SCC lies between 80 and 200,000/ml, this indicates that milk comes from a healthy cow (Bailleul, 1983). Cell count levels varied according to breeds, age and lactation stage. They also varied among regions (Harmon 1994; Rupp *et al*, 2000).

When the SCC is between 200 and 400,000 /ml we can regard milk as suspect. The results of the average value of SCC as well as the enzymatic activities for each milk class are consigned in Table 1. We noted that more than 60% of the milk samples belonging to class 1 have a rate of average somatic cells of 150,000/ml with a minimum of 21,000 and one maximum of 196,000/ml.

Table 1.: Classification of milk according to SCC,NAG-ase activity and fluorescence

	Class 1	Class 2	Class 3
SCCX1000/ml	< 200	200-400	>400
%	61,5	25	13.5
Average	150	264	680
NAG-ase (nM/ml/min)	9, 13	12, 1	27, 3
Units of fluorescence	20, 5	26, 25	57

Class 1:

The NAG-ase activity of milk of class 1 determined by our method lies between 3.2 and 18 nM /ml /min., with an average of 9.13 nM /ml/min. These results are relatively higher than those of Kitchen *et al*, (1978) which bring back an average of 5.3 nM/ml/min. 80 % of this class has an enzymatic activity lower than the average of the NAG-ase activity of class 2 which is 12.1 nM/ml/min., with a SCC lower than 300,000 cells /ml.

Class 2:

Milk of class 2 presents variable activities ranging between 4.6 and 19.2 nM/ml/min. Variability is less important on the level of the SCC, which concentrates between 200 and 300,000/ml.

Class 3:

This class is characterized by strong enzymatic activity and higher than 24.6 nM/ml/min. in almost the totality of the samples. The corresponding number of somatic cells in all classes of milk lies between 500 and 900,000 cells/ml.

These results show that it is possible to classify milk according to its degree of infection while being based only the activity of NAG-ase. We thus propose the classification that follows:

Normal: < 12 nM/ml/min.
Suspect: 12 – 24 nM/ml/min.
Anormal: > 24 nM/ml/min.

We evaluated the activity of NAG-ase in milk of mixture directly from the farm. The found values lie between 9.4 and 17.8 nM/ml/min. It should be recalled that the age and stage of lactation should be taken into account.

Conclusion

The procedure of the NAG-ase activity analysis proposed is a simple, rapid and efficient method. It could be used for routine analysis such as mastitic milk detection and diagnosis. Compared to the fluorimetric method, this method gave practically the same results but did not require a more sophisticated instrument because it uses a molecular absorption spectrometer read in the visible region. The preliminary steps of precipitation, centrifugation or filtration are unnecessary. The sensitivity of the two methods is comparable while the turbidity could lead to the conclusion that the two enzymatic methods are applicable in the control of milk quality.

References:

Bailleul, B (1983) Leukcyte counts in milk. *La technique laitière.* **974**: 44-46.

Chassagne, M., Barnouin J. , Chacornac J.P. (1998) Biological predictors for early clinical mastitis occurence in Holstein cows under field conditions in France. *Prev. Vet Med.* **35**: 29–38.

Choukri, A., Humbert, G., Guingamp, M.F., Linden, G. (1990) Utilisation of synthetical substrale and dissolving reagent for determination of lipase and NAG-ase activity. Briefs, *Communication and Abstracts of Posters of XXIII International Dairy Congress*, Montreal, **1**: 23.

Choukri, A (1991) *Rapid control of milk and dairy products quality: utilisation of dissolving reagent for determination of enzymatic activities and thermic traitement appreciation.* Doctorat. Thesis, Univ. of Nancy, France.

Choukri, A. (1997) The spectrometry in analysis and control of milk and milk products quality: principle and application. *National Seminar on Food Quality Control,* SBA, Algeria, p. 7.

Emanuelsson, U.T, Olsson, T. Holmberg, O., et al (1987) Comparison of some screening tests for detecting mastitis. *J . Dairy . Sci.* **70**: 880-887.

Guingamp, M.F, Humbert, G., Choukri, A., Linden, G. (1989) Transparization technique of milk and milk products. *Process* **1036**: 35-39.

Harmon R.J (1994) Physiology of mastitis and factors affecting somatic cell counts. *J. Dairy Sci.* **77:** 2103-2112.

Kearjean, J.R., Dousset X., Blaise, P (1986) NAG-ase activity evaluation for the somatic cell count in bovine milk. *IAA* 273-276.

Kitchen, B.J., Middleton, G (1976) Enzymatic method for determination of the somatic cell count in bovine milk: II. NAG-ase test for the routine estimation of the somatic cell count in milk. *J. Dairy Res.* **43**: 491-494.

Kitchen, B.J., Middleton, G., Salmon, M. (1978) Bovine milk NAG-ase and its significance in the detection of abnormal udder secretions. *J. Dairy Res.* **45**: 15-20.

Kitchen, B.J. (1981) Review of the progress of dairy science: Bovine mastitis: milk compositional changes and related diagnosis tests. *J. Dairy Res.* **48**: 167-188.

Maisi, P., Riipinen, I. (1988) Use of California mastitis, NAG-ase and antitrypsin to diagnose caprine subclinical mastitis. *J. Dairy Res.* **55**: 309-314.

Nagahata, H., Saito, S. Noda, H. (1987) Changes in NAG-ase and b- glucorinidase activities in milk during bovine mastitis. *Can. J. Vet. Res.* **51**: 126-134.

Obara, Y. (1985) Diagnosis of bovine mastitis by determination of lysosomial enzyme activity in milk. *J.A.R.Q.* **4**: 298-304.

Pyorala, S. (1988) Indicators of inflammation to evaluate the recovery from acute bovine mastitis. *Res. Vet. Sci.* **45**: 166-169.

Rupp. R., Boichard D. Bertrand, C., Bazin, S (2000) Overview of milk somatic cell counts in the French dairy cattle breeds. *INRA. Prod. Anim.* **13** (4): 257-267.

Rupp. R., Boichard D (1999) Genetic parameters for clinical mastitis, somatic cell score, production, udder type traits, and milking ease in first lactation Holsteins. *J. Dairy Sci.* **82**: 2198-2204.

Serieys, F., Auclair, J., Poutrel, B. (1987) Influence of mammary infections on milk chemical composition. *C.E.P.I.L, INRA, Fr.*161-170.

(Received 16/10/2000 , in revised form 28/02/2001)