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Enzymatic Determination of Somatic Cells by Using Transparisation in Raw Milk

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Abstract

The transparisation technology for milk and milk products could be applied widely and very importantly to various determination because transparisation can economize the cost and increase with precision in the milk payment system. Component of butanone or Triton in transparisation solvent would inhibit the growth of bacteria and method. Enzymatic determination of leucocytes were proposed to evaluate milk quality as mastitis in the milk payment system, this can be easily applied to simplify automation of the determation with the lowest investment cost in milk pay system. The significance of this technique, it can be used in the quality control of raw milk and milk products, milk payment system, and programming of national dairy project. Transparisation technology is used in somatic cell counting by enzymic methods. The range of deviation for this method is 16% in 74 samples. But the deviation is increased to 20% when the Infoss method is used. It is affected by the percentage of epithelial cells and white blood cells in somatic cells from different animals and the stages of aging. NAgase activity has an obvious correlation with white - blood cells in milk. In the case of mastitis the white-blood cells is 90~95% in somatic cells in milk, it is showing greater precision in measuring the state of mastitis. In conclusion the enzymic method of somatic cell counting is a relatively simple and easy method of measurement and can be easily practiced. And the importance of this method is also worth utilizing for indirect counting of Somatic cells by use of synthetic substrates to NAgase. In the future, with the further development of the research in this field, it will be possible to automatize the measurement.

Key words : physical properties, sensory properties, pork cutlets, cured, massaged

Introduction

The milk is synthesized from proscuser which was transported by blood at mammary cells of udder to compose 98% of a new, high molecular substance. We know that milk is the only body fluid which has homogeneous dispersion and it has similarities with blood. But the physicochemical composition is obviously different.

Milk fat is spheric and there are many fat globule membrane with hydrophilic grouping, known as emulsification on its surface. The casein is a big micelle which is composed by a polymerization of α, β, with κ as a protective colloid (Lee et al., 2002).

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The spectrophotometry could be used in various manifestations of this substances if turbidity of caseins or fat could be measured more precise or without complications.

The reason being that all enzymes in milk exist on the surface of the fat membranes sphere, when fat and protein are dissolved and completely dispersed, it is possible to measure the absorbance of the enzymatic reaction by using chemical reagent and the degree of transparency.

There are many kinds of somatic cells in milk, but what we usually refer to are leucocytes. We know that leucocytes are composed of 90~95% in total somatic cells and its their total number is very important for the clinical and hygienic condition of cow and milk. The best way to determine the condition of the udder is according to the number of somatic cells. When the amount of somatic cells is high, it suggests that the udder is in poor condition (Park, 1995).

There are several methods for using fluorescent material in somatic cell counting, such as SEM which uses direct coloration,

Rolling Ball Viscometer (RBV) method utilizing the viscosity of milk, Fossomatic and Somacount methods which color the somatic cells with Ethidium bromide, and the Cobra method.

But these methods are time consumption with low accuracy, expensive equipment, and tend forward high cost because of maintenance of instrument, reagents and unanticipated problems etc.

Materials and Methods

Materials

1) Raw milk

The samples of raw milk from 74 different farms were supplied by DM Food Korea Co. Ltd.

2) Reagents

p-nitrophenol, p-nitrophenol· N-acetyl-β-D-glucosaminde, glycine were purchased from Sigma Chemical Co. and other reagents such as NaOH (Yakuri Pure Chemical, Japan), EDTA (Sigma, USA), butanodine (Junsei Chemical Co, Japan), SDS (Bio-Rad, USA) and Oxtoxynol (Junsei Chemical Co, Japan).

Methods of Analysis

1) Determination of SCC by Somacount 300 (Bently, USA)

Determination of procedure was followed according to operation manual routinely.

2) Determination of SCC by enzyme

The measurement of NAgase activity was done by the method of Humbert et al. (1996) with some modification.

(1) Standard curve with p-nitrophenyl : 0.2 mL p-nitrophenyl or standard solution and 0.3 mL substrate solution (5 mM p-nitrophenyl N-acetyl-β-glucosaminide in 0.33 M Na citrate buffer, pH 4.6) are put into a test tube. After incubation at 50℃ for 20 min. The test tube was well shaken after each reagent addition, added 1.5 mL stopping solution (1V 2% EDTA pH 10, 2V 1M glycine NaOH pH 10) and then added 3.5 mL of clarfying reagent (Prolabo, France). The reactional mixture was transferred to cuvette after standing for 2~4 min at room temperature. The absorbancy was then read against a blank in a spectrophotometer (Optizen 2120UV, Korea) at 410 nm within 20 min.

(2) Determination of NAgase activity for raw milk : This was carried out with the same standard process described above standard. In order to stop the reaction of the enzyme with synthetic substrate, the stopping solution was added by increasing to from pH 4.6 to 9.5, as this would prevent the reaction of enzyme during measurement of absorbance.

(3) Change according to the reaction of setting times and temperature were determined with the same mode above. This was done to determine if there were differences of stability of color between time and temperature.

(4) The milk samples were used to determine the changes in NAgase activity according to storage in refrigerated temperatures. This was determined using the same process as above.

(5) The standard curve of enzyme was plotted according to the results using the same mode above, but with NAgase instead of nitrophenol.

(6) For the precision of methods we added supplemental pure enzyme to raw milk and plotted using the same standard.

(7) The number of somatic cells in the serum of a healthy milking cow were also counted in the mechanical counts and compared with NAgase activity determined by the same mode above, to allow for comparison between the milk and serum.

In this experiment we determined the white blood cells by using ABC CBC (ABC vet, 011ab7448, Diagmostics, France). It was based on the principle that the red blood cell and white blood cell (leucocytes) show different refractive index.

Results and Discussion

The best policy for the success of dairy industry is to win the war with somatic cells. The eradiction of mastitis and mastitic milk has many technical disadvantages including an adverse effect on the final dairy product.

For these reasons the detection of mastitis milk is very important for the milk payment system and management of dairy herds in a herd of national or international plan.

This is why endorse following the enzyme determination for numeration of somatic cells with simple, and accurate methods, without use of complicated and expensive machinery.

N-acetyl-β-d-glucosaminide + N-acetyl-β-d-glucosamindase
(synthetic substrate) (enzyme originated from leucocytes
(end products) in raw milk) →
p-nitrophenol

The quantity of liberated end product p-nitrophenol will suggest the activity of N-acetyl-β-d-glucosamindase (NAgase) which can then estimate indirectly the corresponding number of somatic cells in normal and mastitic milk.

For this solution we plotted the optical density at 410 nm with different concentrate of nitrophenol as shown in Fig. 1.

Purified commercial NAgase (Sigma, USA) was standardized with different concentration of nmol in the medium of sterilized raw milk, as indicated in Fig. 2, with R^2=0.9987 over the wide concentration range.

This allows for the possibility that there are no determination problems in a wide range of cell number.

Two types of standardization of NAgase by end product and known enzyme were passed through zero in the graph of standardization and have the same slopes.

It was measured on the optimum temperature for maximum optical density at different degrees from 20 to 100, and from this experiment the maximum coloration should be reach at 50℃ for 20min (Fig. 3). The results are same with ones of Kitchen (1976).

The stabilization of developed color at 37℃ was measured until 90 minutes. From the curve (Fig. 4) measurement, optica density should finish before 20 min which also agreed with the result of Humbert et al. (1996).

In the determination procedure for milk payment the stability of the enzyme is important, since the NAgase activity should not be various according to storage time as it may hinder judgment of raw milk quality.

We have selected 3 levels of cell numbers, 1,145,000/mL, 414,000/mL and 1,122,000/mL (Fig 5). After letting it stand

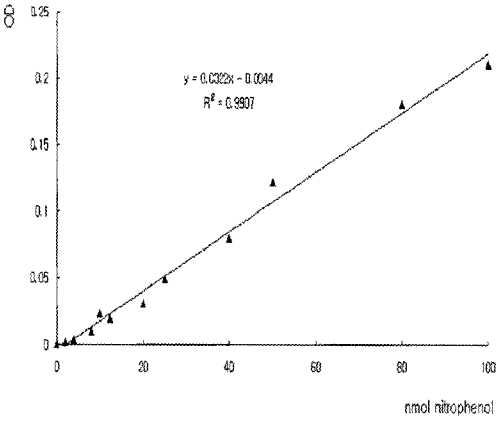


Fig. 1. The standard curve of the nitrophenol.

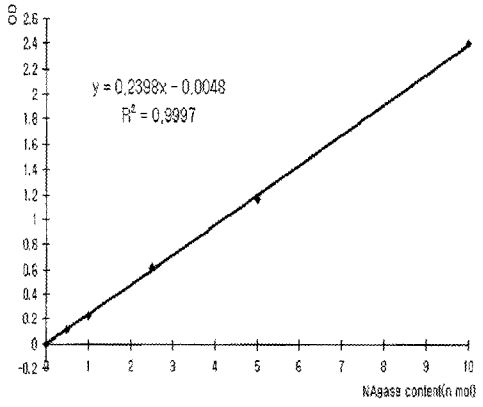


Fig. 2. Standardization of NAgase.

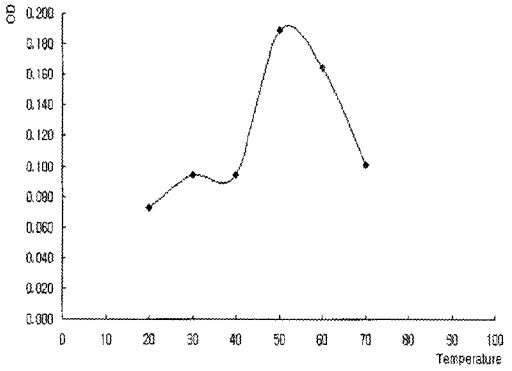


Fig. 3. Optimum temperature for maximum OD at different temperature.

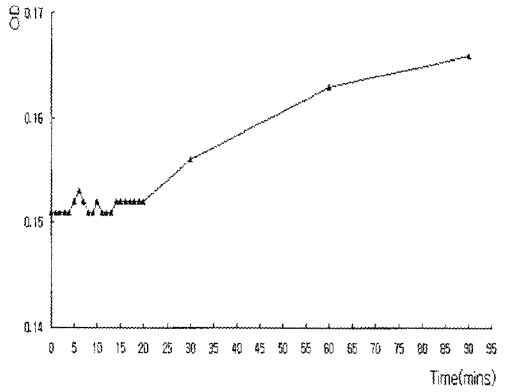


Fig. 4. Change of optical density according to setting time for developed color stability.

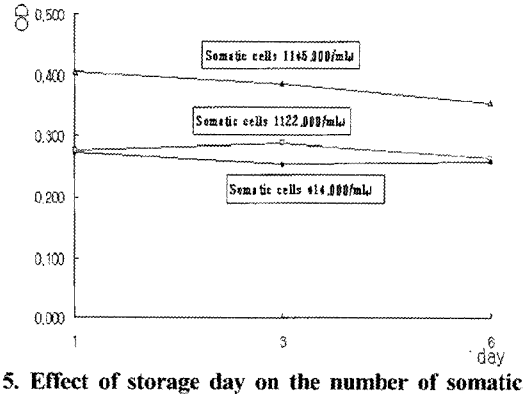


Fig. 5. Effect of storage day on the number of somatic cell counts.

until 6 days at 4℃, there was no change in the original optical density so that storage days could not inhibit the determination within 6 days.

We have been attempting another type of standardization that purifies. Commercial NAgase (sigma. USA) were added to raw milk at different ηmol such as 0.5, 1.0, 2.0, and 5.0 ηmol which rephrased the original amount in raw milk, which added amounts and correction will with amounts of added amounts (R^2=0.9987) but regression line could not pass zero so minus axis indicated the original amounts. Therefore present standardization confirmed the accuracy of this determination (Fig. 6).

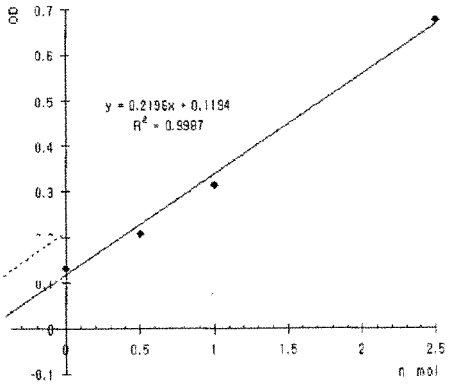
We have attempted to compare the determination of enzyme activity between milk and the serum of a healthy cow according to different dilution. The enzyme activity of number of somatic cells in raw milk correlated well with enzyme activity (▲) such as seen Fig. 7 and the enzyme activity of serum with (■) different dilution did not show a good correlation (deviation A). This was probably caused by interference of transmitted light according to leucocytes concentration.

The difference in sensitivity (B) against cell number in raw milk (▲) and serum (■) could originate from difference of mechanical measuring in Somacount 300 (Bentley, USA) and ABC CBC or from composition differences in milk and serum.

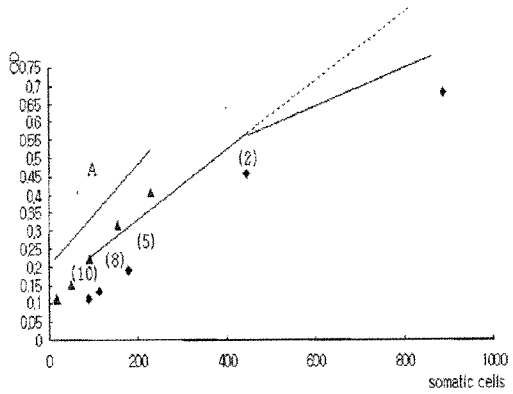
From the above mode of 7 standardization (Fig. 1 to 7) we plotted optical density as an activity of NAgase (Y) of 74 samples in raw milk to the determination of somatic cells, (X) in Somacount 300 (Bentley, USA), which is represented in Fig 8 as R^2=0.7726. Former research also finds that R^2 is 0.84 by Kitchen, et al. (1980), 0.72 by Obara and Komatsu(1984) and as 0.78 by Kerjean et al. (1986), which suggest, the 16% of sample deviation is always from regression line (○: 16%).

In the automatic non destructive determination by ethidium bromide as fluorescent material there were several types of cells such as leucocytes or other mammary epithelium cells which did not release the NAgase counted with leucocytes so there are some errors because of binding capacity difference between cells. It is inevitable that there will be about 16% of errors if we accept the variance of cell in mastitis milk.

In this discussion we should been in mind that instrumental determination has also 16% errors and leucocyte is more important than other mammary epithelium cell and number of leucosis is a more typical sign of mastitis than others, so this determination by enzyme would possible more prec- simple than costly with burden of investmer-

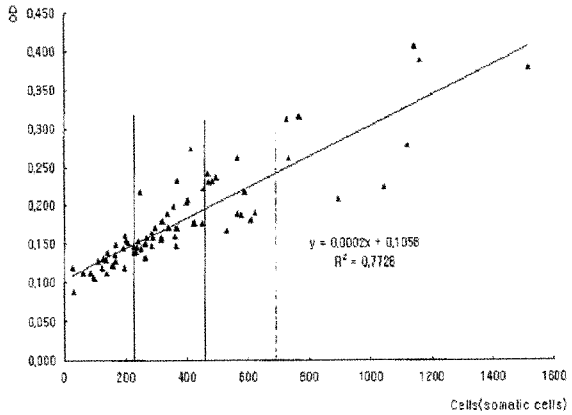


of NAgase addition on raw milk for lization.



(▲) NAgase activities for Somatic cell counts of raw milk measured automatic in ABC CBC.
(■) NAgase activities for Somatic cell counts of healthy cows serum with different dilution times.
( ) is dilution concentration.

Fig. 7. Comparison of optical density of NAgase between raw milk and healthy cows serum depending on different dilution.



/: grade 1(220,000), /: grade 2(460,000), /: grade 3(700,000)

Fig. 8. Correlation between somatic cells and optical density at raw milk (y=0.0002x+0.1058, n=74).

particularly, in developing countries for the system of milk payment in future.

Furthermore, this method can be easily to simple automatic determination with the lowest investment cost in milk payment system.

Acknowledgments

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