A rapid method for determination of somatic cell count and isolation of neutrophil elastase from bovine milk

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Abstract: *A rapid method for SCC with YOYO1 was developed. Dying the cells fluorescent is an advantageous technology with accurate and repeatable results. PMN elastase from bovine milk was obtained and proven by a RP-HPLC. The activity of the PMN elastase was measured and compared with porcine pancreatic elastase. Measuring the PMN elastase activity through created method is helpful for determination of the cattle health.*

Key words: *SCC, mastitis, PMN elastase, elastase activity, YOYO1, HPLC, milk.*

INTRODUCTION

Milk production is a main branch in many countries. Mastitis is one of the biggest problems for farms. The limit point of somatic cell count (SSC) is 400 000 cell/mL according to Regulation EC № 853/2004. Animals with more than this count are classed as mastitis infected. There are fast tests for mastitis but they are insensitive. Veterinarians and firms that redeem the milk need a fast and correct method to establish the disease. Preventing mastitis is essential for owner of dairy animals.

The cattle with mastitis symptoms have changes in the blood and milk cells. Daniel Schwarz et al. [8] explain that the quantity of polymorphonuclear neutrophils (PMN) increases in animals with subclinical and clinical mastitis. Extremely low SCC values might indicate problems with immune response [2]. It is a normal response of the body when there is a risk for the health. PMN form the first line of defense against pathogens. They have polymorphic-segmented nucleus and numerous cytoplasmic granules that provide substances for killing bacteria [6]. PMN elastase is located in azurophilic granules. This enzyme is from the class of proteases (serine endopeptidase) which proceeded phagocytosis. It is an enzyme typical for neutrophils [10]. High elastase activity indicates inflammatory and numerous PMN cells.

The purposes of this study are creating a rapid method for SCC in milk and isolation and proof of PMN elastase.

MATERIALS AND METHODS

Reagents and Chemicals

YOYO1 was submitted from Life Technologies (USA). Triton X-100, HPLC-methanol, trifluoroacetic acid (TFA), Tris, Tween, sodium acetate, NaCl, HCl, porcine pancreatic elastase and N-succinyl-Ala-Ala-Ala-p-nitroanilide were delivered from Sigma-Aldrich (Germany). Water was purified with ELGA PURELAB Option.

Milk somatic cell count by fluorescent dye

Milk was collected from two cows grown according to the EU regulations (Regulation EC № 853/2004). They were at the same age and the same lactating. One was clinically healthy and the other one had mastitis symptoms.

Raw milk was tested for SCC with YOYO1 fluorescent dye. Milk samples were kept at 4°C during the isolation procedure. Somatic cells were isolated by centrifugation of 10 mL milk at 200 x g, 10 min, 4°C. The fat was removed and the pellets were collected. Then they were suspended in 5 mL phosphate buffer saline (PBS) (50 mM, pH 7.4). The suspension was centrifuged at 200 x g, 15 min, 4°C. The washed pellet was resuspended in 1mL PBS, vortex mixed and transferred in a vial. Then 10 μL cell suspension were added to 10 μL dying solution (0.1 mg/mL YOYO1 and 1% Triton X-100) [3]. Samples were surveyed with Olympus BX51 fluorescent microscope and 450 nm laser, equipped with QImaging Retiga 2000R camera. Cell counting was made by ImageJ/Fiji 1.46 (http://rsbweb.nih.gov/ij/docs/guide/146-2.html).

PMN isolation from milk

Isolation was performed as per method described by Mehrzad et al. [3]. Briefly, 1 000 mL mastitic milk (somatic cell count 790 000 cells/mL) was diluted to 60% with cold Dulbecco's PBS (pH 7.2) (v/v). PMN were obtained by 3 centrifugations steps. The diluted milk was load in plastic centrifuge tubes and centrifuged (600 x g, 15 min, 4°C). Fat was carefully removed. Supernatant was centrifuge at the same conditions to get better yield. Then the obtained supernatant was separated for further treatment (for elastase isolation). The pellets were washed twice in cold Dulbecco's PBS at 300 x g for 10 min and 200 x g for 15 min at 4°C. Finally, the remaining pellets were suspended in extraction buffer (0.02 M Tris, 1 M NaCl, 0.05% Tween, pH 8.5) [9].

Separation of elastase out of the neutrophil cells (Elastase 1)

The last supernatant was spin at 18 000 x g, 20 min, 4°C. Pellets were discarded, and the liquid over them (hereinafter referred to as elastase 1) was collected and then concentrated by means of evaporator Büchi (Switzerland).

Extraction of elastase in the neutrophil cells (Elastase 2)

PMN pellets were processed by the method of Stoll [9] with some modifications. PMN pellets in extraction buffer were homogenized by a ball mill (IKA Ultra-Turrax Tube Disperser) with maximum speed for 3 min. Then the suspension was frozen (-20°C, 180 min), thawed at room temperature and sonificated for 15 min. This procedure – freezing, thawing and sonification was repeated three times. Thereafter the suspension was centrifuged at 18 000 x g, 20 min, 4°C. The pellet was discarded. The supernatant was contained PMN elastase. Therefore the supernatant was loaded in ultrafiltration cell with membrane cut off 10 kDa (Sartorius, Germany) and 3 atm pressures. During the filtration was added buffer, containing: 0.05 M sodium acetate, 0.05 M NaCl, pH 5.0, thus was made the buffer exchange. Elastase solution (elastase 2) was concentrated with the evaporator.

Elastase proving by RP-HPLC analysis

The reverse phase high-performance liquid chromatography (RP-HPLC) with Exformma PP with Zorbax 300SB C8 (5μm, 9.4 x 250 mm) was used for elastase proving. The eluent was aqueous – organic gradient (water – methanol) and pH was adjusted to 3.0 by adding TFA. The EX1600UV detector was tuned of 220 nm wavelength. Flow rate: 1 mL/min. Injection volume was 5 mL (with 500 μL loop). The sample was 10% and was diluted with 50 : 50 methanol : water [11]. The parameters of the gradient were: 0 – 5 min methanol concentration increases 0 – 14.5% methanol; 5 – 50 min methanol concentration increases 14.5 – 73.15%.

Elastase activity assay

Elastase activity was examed by a colorimetric assay using N-succinyl-Ala-Ala-Ala-p-nitroanilide as specific substrate, measured at microplate reader Rayto RT-2100C (405 nm wavelength). In this assay, 50 μL of the sample was added to 100 μL substrate solution in Tris – HCl (0,1M; pH 8), at room temperature [1, 5]. The concentration of the substrate in this solution is 2 mg/mL. N-succinyl-Ala-Ala-Ala-p-nitroanilide is a specific substrate for elastase. The enzyme cleaves Ala-p-nitroanilide bond and the reacting mixture turns yellow. This reaction is shown in figure 1.

$$SucAla_3\text{-}pNA + H_2O \xrightarrow{\text{Elastase}} SucAla_3 + pNA$$

Fig.1 The enzyme-substrate reaction of elastase and N-succinyl-Ala-Ala-Ala-p-nitroanilide

RESULTS AND DISCUSSION

Milk somatic cell count by fluorescent dye

Somatic cells were centrifuged in low speed to prevent disrupting the cells. They settled on the bottom of the vial and the remaining suspension contains milk lipids and proteins. Fluorescent dye YOYO1 is very bright cyanine fluorochrome with DNA and RNA

specificity. The somatic cells were made permeable with Triton X-100. The used concentrations were to stain all cells. YOYO1 dyed cells were counted on a fluorescent microscope. Samples were in two groups: mastitic milk and non-mastitic milk. Five pictures of each group were made and then was calculated the average number of somatic cells. Two of these pictures are shown in figure 2. We calculated SCC in both milk samples. They were 877 000 cells/mL in mastitic milk and 390 000 cells/mL in milk from healthy cow.

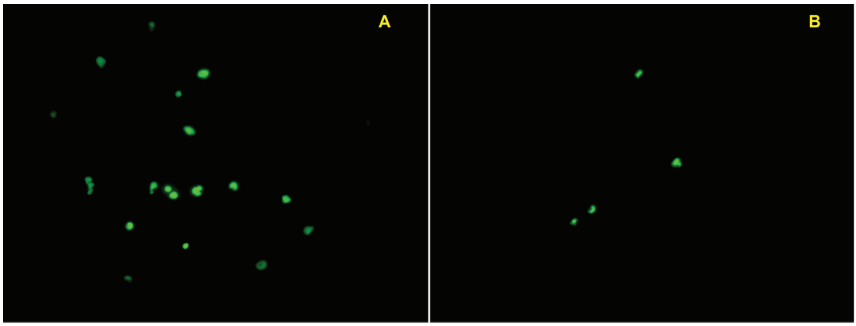

Fig.2 Somatic cells dyed with YOYO 1, magnification 400 x. (A) Mastitic milk with SCC 790 000 cells/mL; (B) Milk from a healthy cow with SCC 360 000 cells/mL.

These two samples of milk were analyzed in accredited laboratory “St. Georgi”, Burgas by standard method – ISO 13366-1:2008 Milk - Enumeration of somatic cells - Part 1: Microscopic method (Reference method). For SCC in mastitis milk they had detected 790 000 cells/mL and in milk from healthy cow – 360 000 cells/mL. Obviously the new method proposed the higher results than standard method, because the new method is more selective.

Isolation of bovine milk elastase

The milk from healthy cattle has low count of somatic cells and these cells are intact, but mastitic cow milk cells are numerous and some of them are destroyed. Mastitic milk was used for further work to investigate the presence of elastase in and out of the neutrophil cells. Low speed centrifuging makes somatic cells settled because they are heavier than other milk components. If there are damaged cells, elastase will be in the supernatant. So the elastase was extracted from both supernatant and pellets. Intact PMN cells were disrupted with extraction buffer and high speed spin, so in this case elastase passed into the supernatant. Ultrafiltration was used for buffer exchange and concentration of the elastase solution. The membrane had cut off 10 kDa, an elastase molecular weight is 33 kDa [9], so it couldn’t pass through the membrane.

Elastase proving by RP-HPLC analysis

An RP-HPLC gradient method was developed to separate and quantify bovine elastase 1 and elastase 2. Certified porcine pancreatic elastase (PPE) was used for comparison with our samples. Figure 3 shows chromatograms of elastase 1, elastase 2 and porcine pancreatic elastase (PPE).

There are 6 similar peaks. №1 and №2 had the highest elastase activity determined by elastase specific substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide, №3 and №4 the middle, №5 and №6 the lowest activity. The elastase molecule has a hydrophilic and hydrophobic part. The chromatographic procedure carried in three steps. The first step was with eluent – water and hydrophilic part of enzyme was eluted. The second and the third steps were carried with 73.15% methanol and the hydrophobic fractions (which distorted from methanol) were eluted [7].

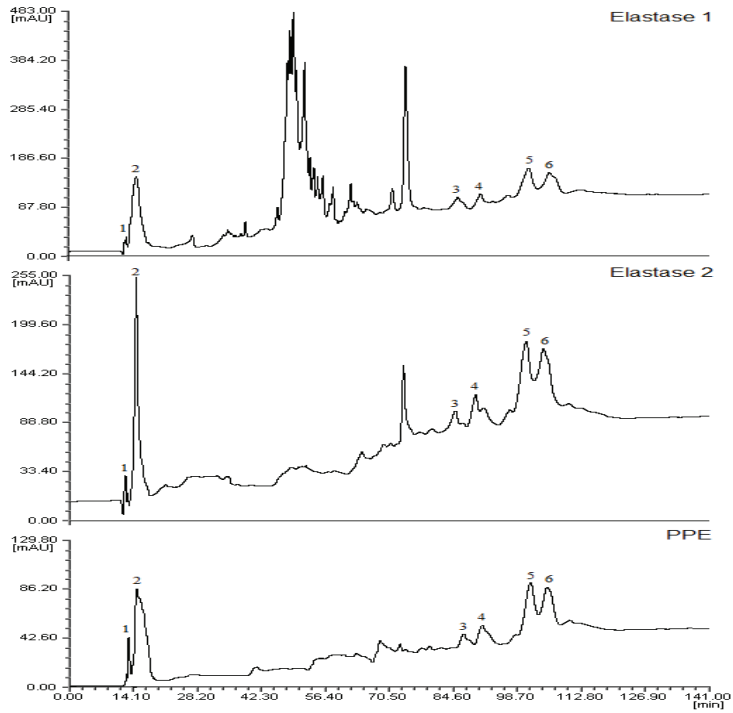

Fig.3 RP-HPLC chromatogram of bovine milk elastase compared with porcine pancreatic elastase.

Elastase activity assay

The obtained chromatographic peaks of Elastase 1, Elastase 2 and PPE were combined as №1+№2, №3+№4 and №5+№6 due to their polarity. Each of them was reacted with N-succinyl-Ala-Ala-Ala-p-nitroanilide as an elastase specific substrate. The absorption of color mixture was measured at microplate reader Rayto RT-2100C at 405 nm (table 1). It was evident, that porcine pancreatic elastase has a higher enzyme activity then bovine milk elastase. Enzyme – substrate mixture with PPE turns yellow immediately, unlike the bovine milk elastase, which needs more than 24 hours to react.

Table 1 Absorption (AU) of color reaction between Elastase 1, Elastase 2 and PPE and N-succinyl-Ala-Ala-Ala-p-nitroanilide as an elastase specific substrate.

	peaks №1+№2	*peaks* №3+№4	*peaks* №5+№6
Elastase 1	1.913	1.770	1.468
Elastase 2	1.943	1.844	1.502
PPE	1.970	1.767	1.611

CONCLUSION

- A rapid method for SCC with YOYO1 was developed.
- Elastase was isolated from the mastitic milk.
- A RP-HPLC gradient was developed for determining the presence of elastase in the pellets and supernatant from the mastitic milk.
- Bovine milk elastase activity was measured and compared with porcine pancreatic elastase activity.

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