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FLUORESCENT MICROSCOPIC ANALYSIS OF BOVINE BLOOD NEUTROPHILS BY QDS LABELED ANTI-BOVINE NEUTROPHIL ELASTASE ANTIBODY

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Abstract: *A reliable method for bovine PMN elastase isolation was performed. Affinity chromatography and gel filtration proved purity of the obtained PMN elastase. This specific neutrophil enzyme was used for anti-bovine neutrophil elastase antibodies obtaining. ELISA tests were made to estimate antibody concentration in blood serum. The obtained anti-bovine neutrophil elastase antibodies had high titer (1 : 6250 serum dilution) with bovine neutrophil elastase as antigen.*

An anti-bovine neutrophil elastase antibody – QDs620nm conjugate was made. UV-Vis and fluorescence spectrophotometric analyses confirmed the successful binding of the components. The obtained conjugate was used for fluorescence microscopic analysis of bovine blood neutrophils. PMN cells were colored brightly with the orange-yellow conjugate and neutrophil cells became visible.

Key words:*bovine PMN, neutrophil elastase, anti-bovine neutrophil elastase antibody, quantum dots, antibody-QDs conjugate*

INTRODUCTION

Blood polymorphonuclear cells (PMN) are the primary guard against disease-causing cells. They have proteases in their azurophil granules which dismembered the foreign cells. PMN elastase is typical serine endopeptidase enzyme in neutrophils. The amount of the PMN cells increase when pathogens enter the body [7]. Therefore, antibodies specific for neutrophils,conjugated with colored component will make neutrophil counting possible. Quantum dots (QDs) are perspective nanoparticles. An antibody-QDs conjugate will color the cells and it will be possible to count them with a fluorescence microscope.

In this paper are described methods for isolation of PMN elastase, anti-bovine neutrophil elastase antibody obtaining, antibody-QDs conjugation and microscopic analysis of bovine blood PMN cells with the conjugate.

MATERIALS AND METHODS

Reagents and Chemicals

SP Sepharose, Sephadex G75, N-succinyl-Ala-Ala-Ala-p-nitroanilide, Complete Freund's Adjuvant, Bovine Serum Albumin, Tween 20, Anti-sheep IgG whole molecule – Peroxydase antibody, 3,3’,5,5’-tetramethylbenzidine, Protein G Sepharose, HiPrep^{TM} 26/10 Desalting, N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride, hydroxysuccinimide, Sephadex G25 Medium were delivered from Sigma-Aldrich (Germany). Quantum dots 620nm were delivered from PlasmaChem GmbH (Berlin).

PMN isolation from bovine blood

Isolation of bovine blood PMN was performed as per method described by Carlson and Kaneko [2]. Isolated bovine blood neutrophils were observed with Olympus BX51 microscope for cell count estimation.

PMN elastase extraction

PMN elastase was isolated from the cells by using a slightly modified version of the procedure of Stoll [6]. The whole process is described in detail in our previous paper [1].

Purification of PMN elastase

The neutrophil elastase extract was loaded on an ion exchange SP Sepharose column (1 x 8 cm) balanced with 25 mmol L^{-1} Na-acetate, 0.2 mol L^{-1}NaCl, pH 7.0 on ÄKTAprime plus instrument. The flow rate was 1 mL min^{-1},fractions of 3 mL were collected. The proteins in fractions were monitored by UV absorbance at 280 nm. A NaCl gradient was applied 0.2-1.0 mol L^{-1} in 143 mL [6].

Neutrophil elastase fractions (fractions 14-17) were concentrated with Vivaspin 20 (10 000 MWCO) and loaded on a Sephadex G75 gel filtration column (1.1 x 55 cm) balanced with 25 mmol L^{-1} Na-acetate, 0.2 mol L^{-1}NaCl, pH 7.0. The flow rate was 0.5 mL min^{-1} and fractions of 2.5 mL were collected [5].

Each fraction was tested for elastase activity by color reaction with specific elastase substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide according to the manufacturer's instructions.

Production of anti-bovine neutrophil elastase polyclonal antibodies

A single sheep was vaccinated with 8 doses bovine neutrophil elastase (1 mg mL^{-1}) with sterile saline (1 mL each). Doses were mixed with Complete Freund's Adjuvant (1 : 1 for the first injection and the others were 2 : 1). The animal experiments were carried out in accordance with *EU Directive 2010/63/EU for animal experiments*.

ELISA screening of antibody production

To estimate the antibody concentration in the sheep blood serum, an indirect ELISA was performed. Each well was coated with 5 μg mL^{-1} pure bovine neutrophil elastase in carbonate-bicarbonate buffer, pH 9.6 (100 μL), and incubated at 37 °C in a shaker for 60 min. The plate was washed 4 times with phosphate buffer saline(PBS) (50 mmol L^{-1}, pH 7.4), 200 μL in a well. Remaining binding sites were blocked with 200 μL in each well of 1% bovine serum albumin (BSA) in PBS. The plate was incubated in a shaker at 37 °C for 60 min again and washed as described above. The antiserum was diluted in PBS + 1% BSA + 0.05% Tween to be tested and 100 μL of each dilution were loaded in the wells. The plate was incubated for 60 min in a shaker at 37 °C and washed as described above. A solution of Anti-sheep IgG whole molecule – Peroxydase antibody in PBS + BSA + Tween (1:10 000 dilution) was added to each well (100 μL). After incubation for 60 min in a shaker at 37 °C and washing as described, the retained peroxydase was visualized by adding 100 μL in each well substrate solution. Five mL of this solution contains 0.5 mL 3,3',5,5'-tetramethylbenzidine (TMB) in dimethylformamide(1 mg mL^{-1}), 15 μL 3% H_2O_2 and 4.5 mL 50 mmol L^{-1} citrate buffer, pH 5.0. The reaction was stopped after 25 min by adding of 2 mol L^{-1} H_2SO_4, 50 μL in each well. The absorption was read with microplate reader Rayto RT-2100Cat 450 nm [4].

Purification of anti-bovine neutrophil elastase polyclonal antibodies

Anti-bovine neutrophil elastase antibodies are IgG type. Affinity chromatography was used for their purification. A column (7x1 cm) with Protein G Sepharose was used. Fractions were 2.5 mL, flow rate 5 was mL min^{-1}. Binding buffer contains 1.732 g Na_2HPO_4 and 1.217 g NaH_2PO_4 in 1 000 mL DI water. Elution buffer contains 3.75 g glycine and 1.744 mL HCl in 1 000 mL DI water.

Then buffer exchange was made for the obtained IgG-fractions with HiPrep™ 26/10 Desalting on ÄKTAprimepluse. Finally, the anti-bovine neutrophil elastase antibodies were in binding buffer and they were lyophilized.

QDs conjugation of anti-bovine neutrophil elastase antibodies

The conjugation procedure was performed as described Hezinová et al, with some modifications.Quantum dots (QDs) 620nm were suspended in PBS (10 mmol L^{-1}, pH 7.4) to concentration 1 mg mL^{-1}. Then another two solutions in PBS were prepared: N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimidehydrochloride (EDC) (8 mg mL^{-1}) and N-hydroxysuccinimide (NHS) (22 mg mL^{-1}). A mixture of 0.5 mL QDs, 5 µL EDC and 5 µL NHS was made and was stirred two hours, protected from light. After that 1 mL anti-bovine neutrophil elastase in PBS (1 mg mL^{-1}) was added. The final mixture was leaved at 4 °C overnight [3].

The obtained conjugate was filtrated with gel filtration and compounds which were not reacted were discarded.A column with Sephadex G25 Medium (28 x 1 cm) was used. Flow rate was 1 mL min^{-1} and fractions were 2 mL each.Absorbance was measured at 280 nm for antibodies and 360 nm for QDs with spectrophotometer JENWAY 6900.

Conjugate fraction spectrum analyses were made with UV-Vis spectrophotometer JENWAY 6900 (for absorption) and fluorescence spectrophotometer F96Pro (for emission).

Finally, the anti-bovine neutrophil elastase – QDs620nm conjugate was lyophilized.

Bovine blood neutrophils counting with anti-bovine neutrophil elastase antibody – QDs conjugate

PMN isolated from bovine blood (see *PMN isolation from bovine blood*) 100 µL were mixed with 25 µL anti-bovine neutrophil elastase antibody – QDs620nm conjugate (1 mg mL^{-1} in 10 mmol L^{-1} PBS pH 7.4). The mixture was stirred at room temperature for one hour. Then 10 µL of the mixture was put on a microscope glass. After completely drying, the sample was observed with Olympus BX51 fluorescent microscope, 365 nm laser, magnification x40 and QImagingRetiga 2000R camera.

RESULTS AND DISCUSSION

Bovine blood PMN were separated from the other blood cells and their amount was determined with microscopic analysis. PMN cells were more than 80% of the total amount of the obtained cells.

Neutrophils have specific protease – elastase, which is in azurophilic granules. Extraction procedure caused the enzyme to release the granules and pass into the supernatant. After that an affinity chromatography was performed for fractionating the supernatant proteins.

Anionic proteins were eluted in an initial peak and cationic proteins were fractionated with the NaCl gradient. Neutrophil elastase is cationic protein and it exits the column after initial anionic protein peak (figure 1).

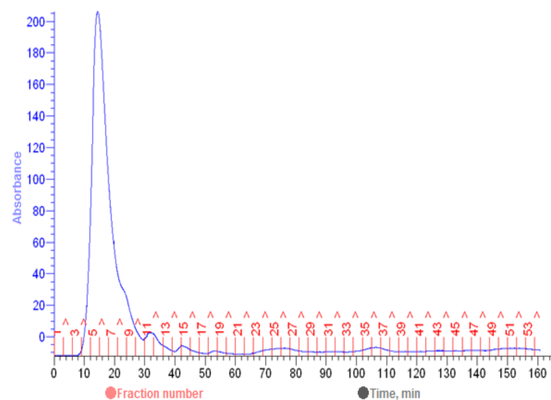

Fig. 1. Affinity chromatography with NaCl gradient for purification of bovine PMN elastase extract

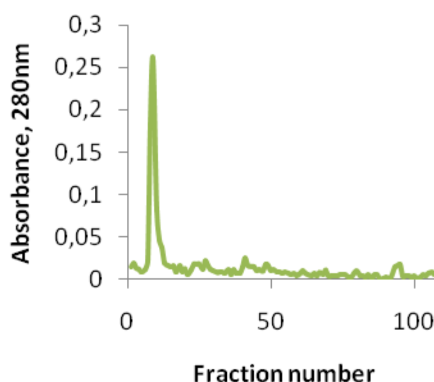

Fig. 2. Gel filtration of the elastase active fractions (14 – 17) after affinity chromatography

Each cationic peak was examined for elastase enzyme activity with specific substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide. Fractions 14 to 17 gave a color reaction which confirmed elastase presence.

Elastase active fractions (14 – 17) were filtrated for size exclusion with Sephadex G25 Medium and obtained peaks were examined for elastase activity. The biggest peak (fraction 8) on figure 2 had elastase activity which proved advantages of the purification method.

The obtained pure PMN elastase was used for vaccination of a sheep. The antibody rich serum from sheep blood was separated. ELISA tests were made for screening of antibody production (figure 3). The linear range of the ELISA test in the middle of the injection period is smaller (1 : 50 to 1 : 1250 dilution) than that after the end of the injection period (1 : 50 to 1 : 31250 dilution) which confirmed high sensitivity of the anti-bovine neutrophil elastase antibodies.

Anti-bovine neutrophil elastase antibodies are IgG type, so they were taken from the other antibodies in the serum by affinity chromatography.

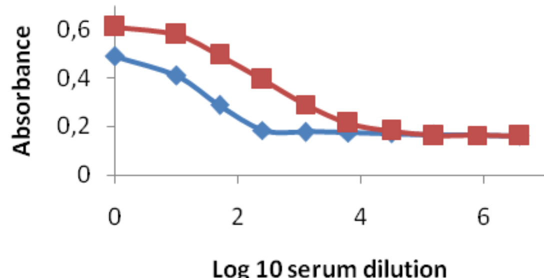

Fig. 3. ELISA of serum anti-neutrophil elastase antibodies before (♦) and after (■) the end of injection period

A conjugate with anti-bovine neutrophil elastase antibodies and QDs620nm was made. Antibodies were added in excess in order to ensure optimal binding to QDs. Figure 4 shows successful filtrating of the conjugate (fraction 5) and the removal of the free antibodies (fraction 8).

Fraction 5 was the conjugate fraction, so spectrum analysis was made. The spectra of fraction 5, free QDs620nm and free anti-bovine neutrophil antibodies were compared (figure 5 and figure 6). The slight shifted spectrum is evidence for linking the components. The UV-Vis spectra of the unbounded anti-bovine neutrophil antibodies showed peak at 268 nm, and the conjugate – at 273 nm (figure 5). The fluorescence measurement showed maintaining the overall shape and ~ 10 nm shift of the anti-bovine neutrophil antibody – QDs620nm conjugate compared with free quantum dots 620nm (figure 6). These were sufficient evidence of the successful connection of the reaction components.

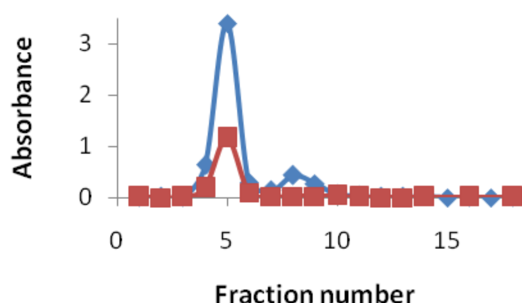

Fig. 4. Gel filtration of the anti-bovine neutrophil elastase antibody – QDs620nm conjugate. Absorbance at 280 nm (♦) for protein presence and absorbance at 360 nm (■) for QDs

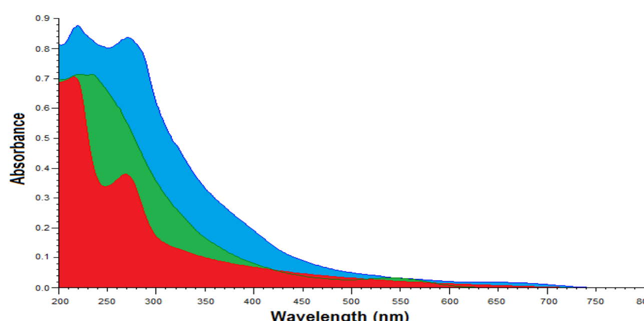

Fig. 5. UV-Vis spectrum analysis of free anti-bovine neutrophil elastase antibody (red), free quantum dots 620nm (green) and anti-bovine neutrophil elastase antibody – QDs620nm conjugate (blue)

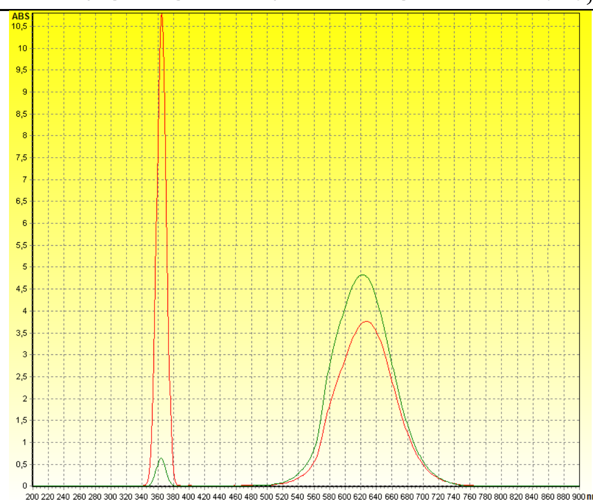

Fig. 6. Fluorescence spectra analysis of free quantum dots 620nm (green) and the anti-bovine neutrophil elastase antibody – QDs620nm conjugate (red)

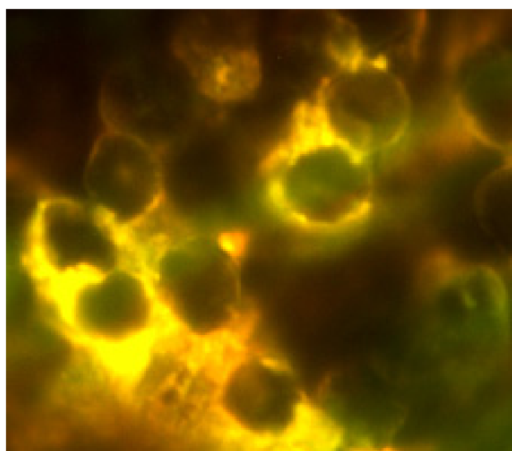

Fig. 7. Bovine blood neutrophils brightly colored with the anti-bovine neutrophil elastase antibody-QDs conjugate

The obtained conjugate was applied in real test with bovine blood PMN cells. Figure 7 shows brightly colored neutrophils with the antibody-QDs conjugate. The conjugate had orange-yellow color which made PMN cells visible.

CONCLUSION

- A purified bovine PMN elastase was obtained.
- Anti-bovine neutrophil elastase antibodies were harvested.
- An anti-bovine neutrophil elastase – quantum dots 620nm conjugate was obtained.
- A fluorescence microscopic analysis of the bovine PMN cells was made.

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