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In vitro production of neuraminidase by *Clostridium chauvoei* (Jakari strain)

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ABSTRACT

The production of neuraminidase (sialidase, EC 3.2.1.18) by *Clostridium chauvoei* (Jakari strain) was investigated and a novel neuraminidase was detected in the bacterium *in vitro* using a fluorescent substrate. Neuraminidase activity was reproducible and had a linear relationship with the amount of bacteria at lower bacterial concentrations. The relationship was no longer linear at higher bacterial concentrations (3.33×10^7 to 4.44×10^7 cfu/ml). The possible role of this enzyme in the pathogenesis of blackleg disease is discussed. It is concluded that the enzyme could be playing the role of spreading the disease in the tissues of infected ruminants. Therefore, the use of neuraminidase inhibitors to manage the disease clinically should be thoroughly investigated.

Key words: neuraminidase, *Clostridium chauvoei*, blackleg

Introduction

Neuraminidases (sialidases, EC 3.2.1.18) belong to a class of glycosyl hydrolases that release terminal N-acetylneuraminic (sialic) acid residues

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from glycoproteins, glycolipids and polysaccharides (ROGGENTIN et al., 1993). These are key enzymes of sialic acid catabolism, hydrolyzing the glycosidic linkage between sialic acid molecules and the penultimate sugar of the carbohydrate chains of oligosaccharides and glycoconjugates. These enzymes have been detected in a variety of microorganisms such as viruses, bacteria and protozoa (MULLER, 1974; ESIEVO, 1979; PEREIRA, 1983; SAITO and YU, 1993; OLADELE et al., 2002). For some microbial pathogens, sialidases are believed to act as virulence factors, allowing successful competition with the host by alleviating their spread in host tissue (GODOY et al., 1993).

Blackleg, which is a disease of cattle, sheep and other ruminants, was first reported in Nigeria by 1929, cited by OSIYEMI, 1975) and has remained a major problem of cattle in the country. Nigeria's epidemiologists classify the disease as a "List A disease" in the country, because of the very high mortality rates associated with it at the onset of the rainy season (ABDULKADIR, 1989). Although vaccination against the disease has been carried out since 1930 (OSIYEMI, 1984), many sporadic outbreaks have been recorded annually. The nomadic Fulani Pastoralists of rural Nigeria who own about 70-80% of total livestock in the country (SULEIMAN, 1988) prefer the use of traditional (herbal) remedies to manage blackleg. Most of them still have reservations about the use of conventional drugs to manage the disease (ABDU et al., 2000). The use of neuraminidase inhibitors to clinically manage infectious diseases, whose aetiologic agents produce neuraminidase, has been advocated (HAYDEN et al., 1997). This study was therefore conducted to investigate the production of neuraminidase by *Clostridium chauvoei* (Jakari strain) *in vitro* to provide a clear understanding on the pathogenesis of blackleg disease and we report here, for the first time, that the bacteria produce neuraminidase.

Materials and methods

Reagents. All the reagents used in this experiment were purchased from Sigma Chemical Company, St. Louis, U.S.A. Media (agar) for bacterial cultivation were procured from Oxoid Company, England.

Bacterial strain. *Clostridium chauvoei* (Jakari strain) was obtained in its lyophilized form from the National Veterinary Research Institute, Vom,

Plateau State, Nigeria for this experiment. The bacterium was isolated from clinically infected Zebu cattle and its pathogenicity indices have been fully determined (PRINCEWILL, 1965).

Media preparation. Three media were used for cultivating *Clostridium chauvoei* (Jakari strain) to assay for neuraminidase activity, namely: reinforced clostridial medium (RCM), blood agar (BA) and cooked meat medium (CMM). The media were prepared by modifying the methods described by DOWELL and HAWKINS (1981) with all procedures for microbiological asepsis strictly adhered to.

RCM was prepared by weighing Bacteriological peptone (1%), meat extract (1%), yeast extract (0.3%), Sodium Chloride (0.5%), Sodium acetate (0.8%), agar base (0.05%), soluble starch (0.1%), L-cysteine hydrochloride (0.06%) and D- Glucose (0.6%), respectively, into a conical flask using a sensitive balance (Medical market®) and with the addition of 100 ml of distilled water. The mixture was autoclaved at 121 °C, 15 pounds pressure, for 15 minutes. The pH of the medium was adjusted to 8.4 after cooling. Ten ml of the prepared RCM was dispensed into sterile bottles and kept at 4 °C to be used on the same day it was prepared.

BA was prepared by weighing 37 g of agar base (powder) into a 2-litre-capacity beaker containing 1 litre of distilled water and allowed to soak for 10 minutes. This was swirled for proper mixing, sterilized as stated earlier and allowed to cool to 47 °C before adding 5% defibrinated sheep blood. The sheep blood was properly mixed with the remaining content of the beaker before pouring the prepared BA onto plates. After drying, the BA was used on the same day.

Commercially prepared CMM was reconstituted by weighing 237 g into a 2.5-litre-capacity beaker and 1.9 litres of distilled water added to it. This was left to soak for 20 min. after which the mixture was autoclaved as described earlier for RCM and BA before being allowed to cool to room temperature. The reconstituted CMM was placed in an incubator and used the following day.

Bacterial cultivation. One glass ampoule of *Clostridium chauvoei* (Jakari strain) was severed using a pair of sterile scissors. Aliquots (0.5 ml) of RCM was transferred from a sterile bottle containing 10 ml of RCM

into the glass ampoule using a sterile 2 ml syringe, rinsed and emptied back into it. This was then placed in an incubator at 37 °C for 48 hours to reactivate the lyophilized bacteria. Thereafter, the reactivated bacteria were removed from the incubator and 4 drops were placed on a blood agar plate using a Pasteur pipette. This was incubated for 48 hours to identify the colonies of *Clostridium chauvoei* (Jakari strain). Pure colonies of the reactivated bacteria in 10 ml RCM were transferred into a 2.5 litre sterile culture bottle containing 1.9 litres of CMM and placed in a gaspak anaerobic jar, whose lid was screwed until it became hand tight. The anaerobic jar and its contents were placed in the incubator at 37 °C for 72 hours. Microbiological asepsis was maintained at each stage of bacterial cultivation.

Serial dilution. One ml of culture supernatant was pipetted and used for serial dilution. A 10- fold dilution was carried out (NORTON, 1986). Nine ml of cysteine-glucose-peptone water (CGPW) was dispensed into sterile McIntire bottles of 20 ml capacity and labelled 1 to 7. The 1 ml of culture supernatant earlier pipetted was transferred to McIntire bottle number 1, mixed well; subsequently, 1 ml was transferred from the preceding bottle to the next up to the 7th sterile bottle. Using a sterile micropipette, 4 drops of the content of bottles number 1 to 7 (serially diluted 10-fold) were transferred to sterile blood agar plates labelled 1 to 7 respectively and incubated anaerobically at 37 °C for 24 hours using gaspak anaerobic jar. The colonies on blood agar plates 6 and 7 were counted and their averages computed and expressed as number of colonies per ml of culture (colony-forming units per ml, i.e. cfu/ml). Each micropipette drop on blood agar plate is equivalent to 50 µl capacity and hence 50 was used as the factor to compute the amount of bacteria (cfu/ml) in the culture. At each stage of the experiment, microbiological asepsis was strictly maintained.

Processing of culture medium. CMM containing the cultivated bacteria was placed in a refrigerated centrifuge (Mistral 4L, MSE) and spun at 9,000 g for 40 minutes at 4 °C. The supernatant was decanted immediately into two sterile plastic containers each of 1 litre capacity. The plastics containing the culture supernatant were kept at -20 °C until required.

Ammonium sulphate [$(NH_4)_2SO_4$] fractionation of culture supernatant. Three ml each of culture supernatant were dispensed into test tubes containing 168 mg, 210 mg, 528 mg, 726 mg, 939 mg, 1.053 g, 1.17 g, 1.416 g and 2.46 g of $(NH_4)_2SO_4$ corresponding to 10, 20, 30, 40, 50, 55, 60, 70, and 80% $(NH_4)_2S0_4$ saturation respectively. These tubes were set in duplicates. The mixture was then homogenized by violent agitation to apparent homogeneity and centrifuged using a refrigerated centrifuge (Beckman, Model J-21 B) at 20,000 g at 4 °C for 30 minutes. The supernatants were decanted using Pasteur pipette, and 1 ml of phosphate buffered saline (PBS, pH 6.8) was added to each sediment. Neuraminidase activity was assayed in the various fractions as described below to determine the presence of enzyme activity and the fractions with the highest enzyme activity. There was enzyme activity in all the fractions, and was highest in the region of 55-70% $(NH_4)_2SO_4$ saturation. The enzyme fractions in this region were pooled as follows for dialysis: Five hundred ml of culture supernatant was added to 175.5 g of $(NH_4)_2SO_4$ in a beaker and properly homogenized as described earlier. This was then spun in a refrigerated centrifuge (MSE, Mistral 4 L) at 9,000 g at 4 °C for 40 minutes. The supernatant was decanted and saved, as A, while 15 ml of 50 mM sodium acetate buffer, pH 5.5 was added to the sediment. A further 51.5 g of $(NH_4)_2SO_4$ was added to the saved supernatant mentioned above and allowed to dissolve properly as earlier described, and spun in a refrigerated centrifuge at 9,000 g at 4 °C for 40 minutes. The supernatant was decanted and finally discarded, while the sediment saved as B was mixed with A. The mixture is the pooled fraction of the culture supernatant at 55-70% $(NH_4)_2SO_4$ saturation containing pooled neuraminidase.

Dialysis of pooled culture supernatant. Thirty ml of this supernatant was pipetted into a 20-cm long and 6-cm diameter dialysis bag and placed in a 2-litre capacity dialysis beaker containing 1.5 litres of 50 mM sodium acetate buffer, pH 4.5. The dialysis beaker and its contents were placed on a speed plate magnetic stirrer at 4 °C overnight against three changes of the same buffer.

Neuraminidase assay. This was carried out using a fluorescent substrate (KLENNEIDAM et al., 2001). Aliquots (50 µl) of the substrate 4-Methyl Umbelliferyl-Neuraminic acid (4-MU-Neu5Ac) at a final concentration

of 0.7 mM in 50 mM sodium acetate buffer, pH 5.5, was incubated with 50 µl of the dialyzed culture supernatant at 37 ^{0}C for 30 min. At the end of the incubation period the reaction was terminated using 0.5 M borate buffer, pH 9.8. The released methylumbelliferone was then measured at 525 nm excitation and 490 nm emission with a multiple Hitachi fluoroscan. For each determination, the spectrofluorimeter was calibrated with pure methylumbelliferone as standard and readings were corrected by subtracting dialyzed culture supernatant and substrate blanks. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 mM 4-MU-Neu5Ac per minute under the described assay conditions. This procedure was repeated 50 times to test for reproducibility.

Results and discussion

A bacterial concentration of 1.11×10^9 cfu/ml *Clostridium chauvoei* (Jakari strain) was obtained after serial dilution. It was observed during the study that the bacteria produced neuraminidase *in vitro* and the neuraminidase was extra-cellular, released in CMM during bacterial growth. The results of amount of bacteria and neuraminidase activity are presented in Fig. 2. There was a linear relationship between neuraminidase activity and amount of bacteria at lower bacterial concentrations. The relationship was no longer linear at higher bacterial concentrations (3.33×10^7 to 4.44×10^7 cfu/ml). This accords with the findings of ESIEVO et al. (1982), who also reported a linear relationship between *Trypanosoma vivax* lysates and neuraminidase activity *in vitro* at lower concentrations of trypanosome lysates. These workers established that the loss in linearity could be due to substrate exhaustion. The culture supernatant was partially purified through ammonium sulphate saturation and dialysis. Neuraminidase activity was assayed in all the $(NH_4)_2SO_4$ fractions (10-80%) and activity was highest between 55-70% $(NH_4)_2SO_4$ saturation (Fig. 1). This region of highest enzyme activity was pooled before dialysis to obtain maximum enzyme activity, and this approach is hereby presented as a model for obtaining maximum neuraminidase activity from *Clostridium chauvoei*.

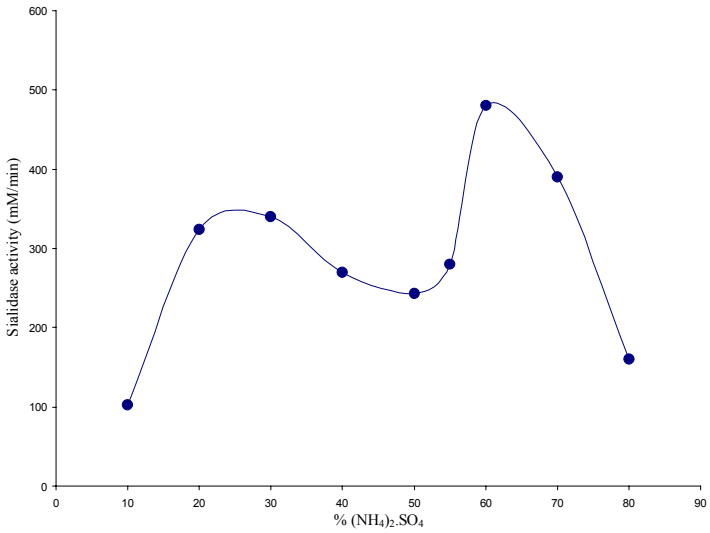

Fig. 1. Ammonium sulphate fractionation curve of *Clostridium chauvoei* (Jakari strain) neuraminidase

The terminal sialic acid from sugar residues and glycoproteins is cleaved by the enzyme neuraminidase, while the detection of the neuraminidase rests on the assay of free sialic acid split from the substrate (WEBSTER and CAMPBELL, 1972; NOK and BALOGUN, 2003). In the present study, a substrate that is specific for neuraminidase (4-MU-Neu5Ac) was used to detect neuraminidase activity in *Clostridium chauvoei* (Jakari strain) because neuraminidase is the only enzyme that cleaves sialic acid from this substrate to produce the classical pink colour that characterises its presence. Based on the report of ESIEVO (1980) that the neuraminidase of *Trypanosoma vivax* cleaved erythrocyte surface sialic acids in clinical infections, it is thought safe to assume that if *Clostridium chauvoei* (Jakari strain) do produce neuraminidase *in vitro*, the same may be applicable if the system is operated *in vivo*. This assumption needs thorough investigation.

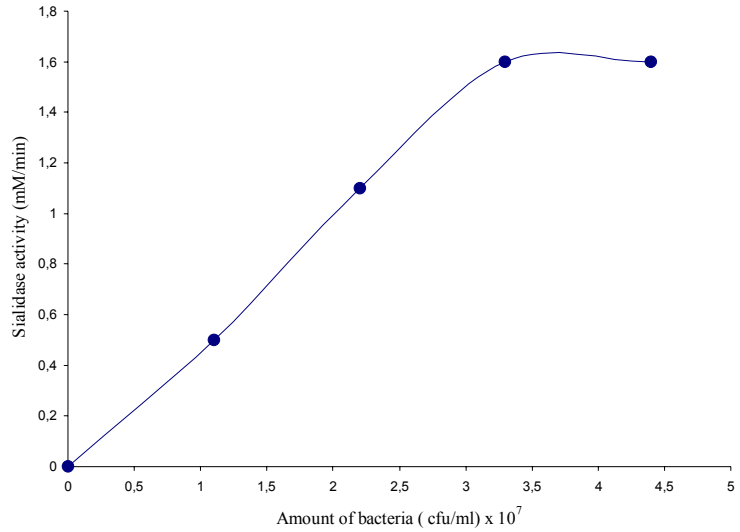

Fig. 2. Expression of neuraminidase as a function of the concentration of *Clostridium chauvoei* (Jakari strain)

In conclusion, this study shows that *Clostridium chauvoei* (Jakari strain) produce neuraminidase *in vitro* and it is therefore suggested that the enzyme could be playing the role of spreading blackleg disease in the tissues of infected ruminants by cleaving their sialic acids to facilitate the spread. It is expedient to clinically assess the therapeutic efficacy of neuraminidase inhibitors in the management of blackleg disease.

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SAŽETAK

Dokazana je proizvodnja neuraminidaze (sialidaze, EC 3.2.1.18) bakterije *Clostridium chauvoei* soja Jakari *in vitro* pomoću fluorescentnog supstrata. Aktivnost neuraminidaze bila je reproducibilna uz linearni odnos količine bakterija pri nižim bakterijskim koncentracijama. Taj omjer nije bio linearan pri višim bakterijskim koncentracijama ($3{,}33 \times 10^7$ do $4{,}44 \times 10^7$ cfu/ml). Raspravlja se o mogućoj ulozi ovog enzima u patogenezi šuštavca. Zaključeno je da bi enzim mogao imati ulogu u širenju bolesti kroz tkiva inficiranih preživača. Uporaba inhibitora neuraminidaze u kontroli bolesti trebala bi biti potpunije istražena.

Ključne riječi: neuraminidaza, *Clostridium chauvoei*, šuštavac