Detection of the Keratinolytic Activity of Agriculture and Mount Barker Strain *Dermatophilus congolensis* Serine Proteases

Hashemi Tabar*[1], G.R., Davies, R.[2]

1. Pathobiology Dept, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

2. Biotechnology Research Group, Murdoch University, Perth, Western Australia

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Summary

Keratinolytic activity has been quantified in *Dermatophilus congolensis* (*D.congolensis*). In this study we tried to determine if there was keratinase within serine proteases from Agriculture (Ag) and Mount barker (MB) strains of *D.congolensis*. Therefore, keratinolytic activities of these strains were tested in the presence of Azocasein and Keratine-azure. The keratinolytic activity of papain, subtilisin and proteinase k have also been tested. The results suggest that the keratinase was absent in the serine protease preparations from Ag and MB strains. The keratinase may have been lost in the concentration of the enzyme preparations. Alternatively, the keratinase may need to be induced by the presence of keratin in the culture medium. Therefore, it seems that there is not an agreement of keratinolytic activity of Ag and MB strains of *D.congolensis*.

Key words: *Dermatophilus congolensis,* enzyme, serine protease, keratinase

Introduction

Ovine dermatophilosis is a skin disease caused by *Dermatophilus congolensis* (*D.congolensis*), an aerobe and a facultative anaerobe, gram-positive bacterium. The pleomorphic bacterium life cycle comprises resistant zoospores and invasive thread-like forms or hyphae. Dermatophilosis occurs in sheep, cattle, buffalo, camels,

*Author for correspondence. E-mail:hashemitabar@yahoo.com

horses, goats, antelope, cat and humans all over the world, especially in tropical and subtropical regions where there are high ambient temperatures and high rainfall (Ellis *et al* 1991, Kaya *et al* 2000, Msami *et al* 2001). The infection in sheep results in a reduction of meat and wool production, lamb deaths, culling of affecting stock, a reduction in the value of sheep and wool skins and an expensive treatment (Hashemi Tabar & Carnegie 2002). The disease has a significant economic impact in many developing countries where animal protein is lacking for most of the human population (Larrasa *et al* 2002). Additionally the lesion caused by a *D.congolensis* infection leaves the sheep susceptible to blowfly strike (Gherardi *et al* 1983). The presence/absence/variation of protease activity levels for *D.congolensis* strains may be linked to the virulence or infectivity of strains. The proteases secreted by isolates with higher protease activity tended to be in the groups with higher infectivity ranking (Ellis *et al* 1993). The serine proteases may have a role in stabilizing the infection within the host (Ambrose 1998). One of the serine proteases secreted by *D.congolensis* may be a keratinase. Since dermatophilosis mainly occur in wool follicle sheaths and skin glands in sheep (Ellis *et al* 1987), and in keratinized tissues in humans (Hanel *et al* 1991) a keratinase may be produced. The keratinolytic activity has been quantified, there was quite significant keratin degradation caused by extra cellular proteases produced by cultures of the bacterium (Hanel *et al* 1991).

The aim of this experiment was to determine if there was a keratinase present within a protease preparation from the Agriculture (Ag) and Mount Barker (MB) strains of *D.congolensis*. These protease preparations were also tested to determine if they were heat stable. Also we tried to test the keratinolytic activity of enzymes such as papain, subtilisin and proteinase k.

Materials and Methods

Materials. The enzymes subtilisin, papain, proteinase K and *D.congolensis* Ag and MB strain serine proteases, and the substrates Azocasein and keratin-azure were

studied. All of the chemicals were purchased from Sigma.

***D. congolensis* strains and culture conditions.** Both the Ag and MB strain serine proteases were obtained from the Department of Agriculture, Baron-Hay Court, South Perth, Western Australia. The strains were isolated from separate ovine field case studies at departmental research stations near Mount Barker, Western Australia. For maximum protease activity, *D.congolensis* was grown in tryptone casein hydrolysate broth (1.0% tryptone [Oxoid], 0.5% NaCl, 1.75% casein hydrolysate [Oxoid] in distilled water, which supplemented with 0.4% sodium glutamate after 5-6 days of culture). The cultures were grown for 2-3 days at 37°C with constant stirring and thereafter at room temperature with constant stirring in closed Schott bottles (1 or 2 Liter). One-ml samples were removed every two days for an Azocasein protease assay. Samples were centrifuged and the supernatants tested. When activity leveled off (highest levels were usually attained at 12-14 days), the culture was centrifuged in 250ml bottles and the supernatant concentrated by dialysis against polyethylene Glycol 20000 (BDH chemicals).

Determination of enzyme activity. 20µl of each strain serine protease was mixed with 180µl sodium phosphate buffer (pH 7.6). The 200µl solution was then added to 800µl of 1mg/ml Azocasein and incubated for 15min at 31°C according to the Lin *et al* (1992) method. All enzyme concentration was 25units/µl, at the time the assay was incubated in the shaking waterbath. For each assay run a set of reference solutions was used as blanks (essentially reagents without the enzyme), comprising 800µl of substrate solution, 200µl of phosphate buffer. The blanks were incubated and treated accordingly depending on the type of assay.

Protease activity assay. 200µl of enzyme solution was added to 800µl of 1mg/ml Azocasein solution, to make a final concentration of 25units/ml. This was mixed and incubated for 24h in a shaking waterbath at the particular temperature. The reaction was terminated with 200µl of 20% trichloroacetic acid (TCA). The control including 800µl of 1 mg/ml Azocasein was incubated at the same condition, after which 200µl

of 20% TCA was added with 200µl of the relevant enzyme solution (the amount that was used as tests in the assays) and thoroughly mixed. The test and control assays were left to stand at room temperature for at least 15min and then filtered. 400µl of the filtrate was added to 200µl of 4M NaOH and mixed. The absorbance was read at 438nm.

Keratinase assay. 200µl of enzyme solution was added to 800µl of 1 mg/ml Keratin-azure solution to make a final concentration of 25units/ml. This was mixed and incubated for 24h in a shaking waterbath at the particular temperature. The reaction was terminated with 200µl of 20% TCA. The control including Keratin-azure was treated as explained above. The test and control assays were left at room temperature for at least 15min and then filtered. 400µl of test or control filtrate was added to 200µl of phosphate buffer and mixed. The absorbance was read at 595nm according to Hanel *et al* (1991).

Ag and MB serine protease rotease's assays at various temperatures. The Ag and MB strain proteases were assayed with Azocasien and Kertain-azure at different temperatures 37°C, 50°C, 60°C and 70°C.

Results

Determination of Ag and MB serine protease's activities. Based on the method of Lin *et al* (1992) one unit of enzyme activity is defined as an increase in the absorbance of 0.001 after the reaction for 15min at 31°C. The Ag strain protease had an activity of 40U/100µl, and the MB strain protease had an activity of 90U/100µl of crude protease extract. The MB protease activity was approximately double of the Ag strain protease activity (Table 1).

Protease activity. In this assay, all enzyme preparations exhibited protease activity at 37°C (Figure 1). Proteinase K and papain exhibited the greatest amount of proteolytic activity at 37°C. The Ag and MB protease exhibited very similar net absorbances. The subtilisin, and papain protease activity increased, and papain were

relatively very high at 50°C. The activity for proteinase K, and the Ag and MB strain serine proteases dropped at 50°C.

Table 1. *Determination of enzyme activity for Ag and MB strain serine proteases*

Strian		Absorbances (438nm)			Average	C of variance*	Enzyme activity	Net gain
Ag	Control	0.344	0.34	0.338	0.341	1.16	40 U/100	0.008
	Test	0.345	0.348	0.353	0.349	0.90	µl	
MB	Control	0.296	0.3	0.302	0.299	1.02	90 U/100	0.018
	Test	0.316	0.316	0.318	0.317	0.36	µl	

*Coefficient of variance(%)=(standard deviation/mean)×100

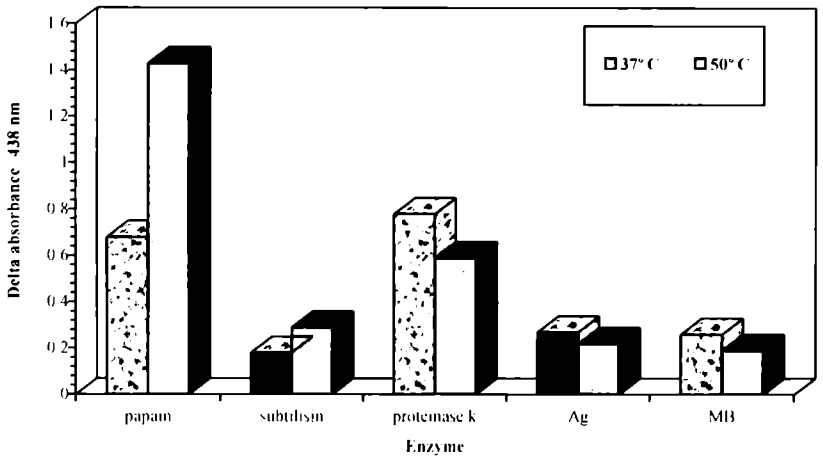

Figure 1. *Enzyme activity with Azocasein at various tempratures*

Keratinase assay. Proteinase K was the only enzyme preparation to degrade the keratin substrate at 37°C (Figure 2). The other enzymes had relatively no keratinolytic activity. The *D.congolensis* strain serine proteases absorbtions were marginally higher than the enzymes that did not exhibit keratinolytic activity, this may not necessarily be significant. At 50°C, the relative activity of proteinase K almost halved. The rest of the enzymes exhibited the same very low levels of keratin degradation. Finally, in the Ag and MB serine protease's assays at various

temperatures, the activity of both the strain protease activities of *D.congolensis* with Azocasien peaked at 37°C and with Keratin-azure the activity of MB strain protease was more than Ag at 37°C. The activities gradually decreased until there was insignificant net absorbances for both proteolytic and keratinolytic assays.

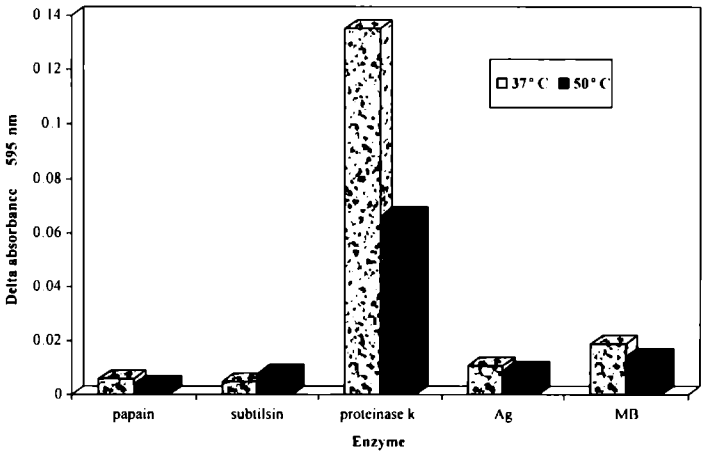

Figure 2. *Enzyme activity with Keratin-azure at various tempratures*

Discussion

Ag and MB strain serine proteases were active against Azocasein. The relative strength of the MB protease was roughly double the strength of the Ag serine protease. Strain variation is a significant factor of *D.congolensis*. Successful vaccination against ovine dermatophilosis has not been achieved and this maybe partly due to the significant strain variations (Ellis *et al* 1991). All enzymes were proteolytically active as indicated by the Azocasein assay. Only the proteinase K was able to degrade Keratin-azure.

Keratinolytic activity has been quantified for *D.congolensis* (Hanel *et al* 1991). Live cultures of *D.congolensis* were incubated for 12 days at 37° C, with only Keratin-azure as a substrate. In some samples there was so much Keratin-azure degradation that it exceeded the capacity of the spectrophotometer to read (i.e.

greater than 2.500). While other samples yielded relatively low keratinolytic activity. The Ag and MB strain serine proteases did not exhibit any keratinolytic activity, when compared to proteinase K, which does degrade keratin. There may be a reason for lack of keratinolytic ability in these strains enzyme preparation, when it has been quantified already in *D.congolensis*. The concentration process of the serine protease may have removed the enzyme from the preparation, i.e. dialysis using PEG 20,000. The keratinase may not have been present in the *D.congolensis* enzyme preparation at all, because the enzyme may have not been induced. Since keratinolytic activity has been quantified from the bacterium (Hanel *et al* 1991), and the results of this experiment yielded more keratinolytic activity, the keratinase may need to be induced. There was no reference to cell growth when the keratinase was induced, consequently whether *D.congolensis* can grow on just keratin as a substrate needs to be determined. The cultures were incubated in a solution with only keratin-azure as the carbon and energy source. During incubation, a keratinase secreted by *D.congolensis* degraded the keratin, which was comparable through the activity exhibited by proteinase K. The Ag and MB strains were grown in a trypton/casein hydrolysate broth, which was absent of keratin. Without keratin in the growth medium, the keratinase was not synthesized by the *D.congolensis* strains. Consequently, the enzyme preparation had no keratinolytic activity for both strains.

In an effort to induce the keratinase from either strain of *D.congolensis*, there are a number of approaches. The technique used by Hanel *et al* (1991) could be duplicated, just simply to observe keratinolytic activity caused by the two strains growth. Alternatively, grow one culture with only keratin as a carbon source and another as described for this experiment, and check the keratinolytic activities of all protease preparations. *D.congolensis* would be tested to see if the bacterium would be able to grow with keratin as the principle carbon source. The induction of a keratinase, if any, should be in the cultures that had only keratin as a carbon source. The Ag and MB serine proteases were tested for their temperature stability. Both

had peak activity at 37° C, and proceeded to decrease as the temperature of the assays increased. If there was no keratinolytic activity in the Ag and MB strain protease preparations, and if the recombinant serine protease sequence was the keratinase then the thermal stability of the keratinase was not tested. Since the keratinase was absent from the protease preparation, other proteases were tested for their thermal stability. If the keratinase is inducible, to determine the heat stability, the protein will need to be induced and then tested. There were some problems in aspects of the experiment. For the keratinolytic assay, the vigorous shaking action of the waterbath produced small clumps of the keratin-azure. This still happened even though the strands were between 2 and 5mm in length. This would surely mean that the solution was not homogenous, and hence only a part of the enzyme was actually available to act on the keratin, yielding a false activity.

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