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Abstract of Dissertation

Applicant

Doctoral Program in Animal and Food Hygiene

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Title: Expression and characterization of digestive enzymes from tsetse (*Glossina morsitans morsitans*)

(ツェツェバエ由来消化酵素の発現と性状解析)

Abstract

Introduction: Tsetse flies are strict blood feeders and are classified into one genus, *Glossina* of the family Glossinidae, order *Diptera*. *G. morsitans morsitans* is solely a daytime feeder widely distributes in the region of tropical Africa having a mean annual temperature of varying from 19-28 °C. Both sexes respond to CO_2 produced by the exhalation of animals and feed exclusively on blood and produce anticoagulants to prevent clotting. The economic and biological importance of the *G. m. morsitans* in the fact that its species are the primary vector of trypanosomes. In particular, *Trypanosoma brucei* and *T. congolense,* which are considered as a major cause of human African trypanosomosis (HAT) or sleeping sickness and animal African trypanosomosis (AAT) or nagana disease. The transmission occurs when a tsetse fly bites an infected animal and contracts the protozoan while feeding off of its blood. The parasites then rapidly move to the mid intestine of the fly and begin to reproduce. From here they move to the fly's salivary gland and remain there to mature. Once in the epimastigote form, they divide and are ready to be transmitted to a mammalian host via saliva injected into the bite. After uptake the blood meal from infected vertebrate, tsetse flies secrete several digestive enzymes into the midgut lumen to digest the blood protein contents and propose to kill the parasites. In the tsetse midgut, trypanosomes must interact with the hostile environment of the midgut lumen, in which proteolytic enzymes and digested products might affect their differentiation and survival. Thus, the midgut is not only the site for synthesis and secretion of digestive enzymes and nutrient absorption, but also a primary interface between tsetse and trypanosome. Hence, the physiology of blood meal digestion may substantially influence trypanosome differentiation and survival in the tsetse fly.

Objective: In order to understand the mechanism of enzymatic activity in blood meal digestion and tsetse-trypanosome interaction. The general objectives of this study are, (1) to elucidate the mechanism of blood meal digestion in the midgut of tsetse, (2) to find out the

method to prevent trypanosome infection in tsetse, and (3) to improve the quality of human health and animal health in Sub-Saharan Africa. The specific objective aims to characterize digestive enzymes from the midgut of tsetse and to investigate their roles on tsetse-trypanosome interaction.

Material and Methods: (1) Digestive tract of male tsetse flies were dissected and produced total RNA. (2) The gene of digestive enzymes namely cathepsin B (GmcathB) and cathepsin L (GmcathL) cysteine protease and trypsin-like serine protease (Gmtrypsin) were cloned from the tsetse midgut. (3) The recombinant proteins of three digestive enzymes were expressed by using bacterial protein expression system (*Escherichia coli*) and yeast protein expression system (*Pichia pastoris*). (4) All recombinant proteins were used to analyze the enzymatic activity toward blood protein contents such as bovine hemoglobin (Hb) and bovine serum albumin (BSA) together with using fluorogenic casein and synthesis specific substrates. (5) Analyze the effect of all recombinant proteins to cultured trypanosomes *in vitro* condition mimicking insect habitat .

Results:

Chapter 1: A DNA fragment (930 bp) encoding pro-cathepsin B (Accession No. AF329480_1) was cloned and expressed in *E. coli* and *P. pastoris* protein expression system. An active recombinant cathepsin B (rGmcathB) produced by *P. pastoris* exhibited the highest proteolytic activity at pH 4.0 and at 30°C. It has degraded bovine hemoglobin and serum albumin at that optimal condition. Moreover, rGmcathB exhibited hydrolysis preference for bovine hemoglobin more than for Z-Arg-Arg-MCA. The proteolytic activity of rGmcathB was inhibited by specific cysteine protease inhibitor (E-64) confirmed belonging to papain-like cysteine protease family. These results indicated that rGmcathB shows the activity of cathepsin B and have high affinity with blood protein referring a role in blood meal digestion. This study has proved that GmcathB was able to kill the parasite at early phase of infection and somehow differentiated phase. The result in this study also implied that *P. pastoris* expression system is more suitable for expressing enzymatically active recombinant proteases than *E. coli* expression system because recombinant protein expressed by *E. coli* system was not active. Moreover, this study is the first report to characterize the enzymatic activity of cathepsin B from tsetse.

Chapter 2: A cDNA encoding a cysteine protease named TC 31 cathepsin L-like protease, which was previously identified in the fat body of adult tsetse, was cloned from a midgut of adult male tsetse flies (48 h post blood meal). The cDNA encoded 516 amino acid residues having the highest identity to cathepsin L of *S. peregrina* (82 %). The amino acid sequence contained conserved amino acid residues, Cys-His forming the catalytic dyad of an active site of C1A cysteine protease family, an Asn orients the imidazolium ring of the catalytic His, and a Gln helps to form the oxylation hole. The recombinant GmcathL (rGmcathL) was successfully expressed by using *P. pastoris* protein expression system. Activation of rGmcathL was occurred in presence of DTT (2.5 mM) and cysteine (10 mM). Proteolytic activity was exhibited the highest at pH 6.0 with broad temperature range from 25 to 37 °C. The proteolytic activity of rGmcathL was completely inhibited by broad spectrum cysteine protease inhibitor (E-64). The catalytic efficiency (k_{cat}/K_M) of rGmcathL was obtained from hydrolysis of Z-Phe-Arg-MCA indicating rGmcathL exhibited hydrolysis preference for amino acid that has aromatic ring and hydrophobic side chain (Phe) at P2 position. Moreover, rGmcathL was capable of degrading both hemoglobin (Hb) and bovine serum albumin (BSA), but it preferred to cleave BSA as compared to Hb. Co-cultivation of rGmcatL

and trypanosome resulted in a reduction the number of BSF, while PCF showed relative resistant to the treatment. The results from this study indicated that GmcathL may involve in bloodmeal digestion and parasite elimination.

Chapter 3: cDNA (681 bp) encoding the trypsin-like serine protease precursor (Accession no. AAF91346.1) was cloned from the midgut of tsetse. The protein (Gmtrypsin) consists of 227 amino acids with a predicted molecular mass of 24.4 kDa and a PI value of pH 7.08. An active recombinant protein (rGmtrypsin) was expressed in *E. coli* exhibited the highest proteolytic activity at alkaline pH 8.0 and at 25-30 ˚C on a dose dependent manner. It had a high affinity to BSA, cleaved Z-Phe-Arg-MCA and BApNA while showing preference for arginine at P1 residues. Proteolytic analysis using BApNA revealed catalytic efficiency (k_{cat}/K_M) of Gmtrypsin 0.6 times lower than that of bovine trypsin (BvT). Moreover, *in vitro* co-cultivated trypsnosomes (*T. congolense*) with rGmtrypsin resulted in a significant reduction of the number of BSFs, whereas PCFs are relatively resistant against rGmtrypsin. The transformation of PCFs into EMFs was not observed within 24 h. The results from this chapter indicated that Gmtrypsin is involved in the blood meal digestion and might therefore be useful in elimination of the trypanosomes in the midgut of tsetse.

Discussion: This study has expressed and characterized three digestive enzymes from the midgut of tsetse namely cathepsin B (GmcathB) and cathepsin L (GmcathL) cysteine protease and trypsin-like serine protease (Gmtrypsin). Although, these enzymes have been identified as tsetse midgut digestive enzymes for a while, their enzyme characteristic have not been analyzed yet. Active recombinant protein of GmcathB and GmcathL were successfully expressed in yeast (*P. pastoris*) expression system. While, active recombinant protein of Gmtrypsin was obtained from bacterial (*E. coli*) expression system. Activity profiling with specific substrate and fluorogenic casein substrate indicated that GmcathB and GmcathL are acidic proteases, whereas Gmtrypsin is alkaline protease. All three recombinant proteins displayed their activity in wide range of temperature, which seem to be in range of the temperature of tsetse habitat (25 ˚C). They have a catalytic efficiency for their specific substrates two times lower than the control enzymes.

Conclusion: The results from this study suggest that GmcathB, GmcathL and Gmtrypsin from the midgut are involved in blood meal digestion and elimination of trypanosome at early phase infection. In addtion, rGmcathB and rGmtrypsin appeared to reduce PCF viability, although the percentage was not as high as reduction of BSF. Assuming, GmcathB and Gmtrypsin may serve as valuable tool for probing the physiology of different developmental forms. This study has proved that *P. pastoris* expression system is suitable for expressing enzymatically active recombinant protein from cysteine proteases. However, further investigation of digestive enzymes localization and involvement of digestive enzymes in transformation of insect stage trypanosome into infective metacyclic from are needed to clarify the tsetse-trypanosome interaction.