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The angiotensin converting enzyme (ACE) inhibitor, captopril disrupts the motility activation of sperm from the silkworm, *Bombyx mori*

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ABSTRACT

Angiotensin I-converting enzyme (also known as peptidyl dicarboxypeptidase A, ACE, and EC 3.4.15.1), which is found in a wide range of organisms, cleaves C-terminal dipeptides from relatively short oligopeptides. Mammalian ACE plays an important role in the regulation of blood pressure. However, the precise physiological functions of insect ACE homologs have not been understood. As part of our effort to elucidate new physiological roles of insect ACE, we herein report a soluble ACE protein in male reproductive secretions from the silkmoth, *Bombyx mori*. Seminal vesicle sperm are quiescent *in vitro*, but vigorous motility is activated by treatment with either a glandula (g.) prostatica homogenate or trypsin *in vitro*. When seminal vesicle sperm were pre-incubated with captopril, a strong and specific inhibitor of mammalian ACE, and then stimulated to initiate motility by the addition of the g. prostatica homogenate or trypsin, the overall level of acquired motility was reduced in an inhibitor-concentration-dependent manner. In the course of this project, we detected ACE-related carboxypeptidase activity that was inhibited by captopril in both the vesicular (v.) seminalis of the noncopulative male reproductive tract and in the spermatophore that forms in the female bursa copulatrix at the time of mating, just as in an earlier report on the tomato moth, *Lacanobia oleracea*, which belongs to a different lepidopteran species (Ekbote et al., 2003a). Two distinct genes encoding ACE-like proteins were identified by analysis of *B. mori* cDNA, and were named *BmAcer* and *BmAcer2*, respectively [the former was previously reported by Quan et al. (2001) and the latter was first isolated in this paper]. RT-qPCR and Western blot analyses indicated that the BmAcer2 was predominantly produced in v. seminalis and transferred to the spermatophore during copulation, while the BmAcer was not detected in the adult male reproductive organs. A recombinant protein of BmAcer2 (devoid of a signal peptide) that was expressed in *Escherichia coli* cells exhibited captopril-sensitive carboxypeptidase activities. Our findings show that the BmAcre2 gene encodes a secreted ACE protein included in the *Bombyx* seminal plasma. In particular, the silkworm ACE protein in the seminal fluid might be involved in the signaling pathway that leads to the activation and regulation of sperm motility.

1. Introduction

Peptidyl dipeptidase A (EC 3.5.15.1) is a Zn^{2+} metallopeptidase that cleaves the penultimate peptide bond at the C-terminus of relatively short oligopeptides, usually smaller than 15 amino acids. In mammals, the enzyme is found in the kidney and the renin-angiotensin system (RAS), and is best known for the role it plays in blood pressure homeostasis by cleaving the C-terminal peptide (His-Leu) from the decapeptide angiotensin I to the active octamer angiotensin II. This cleavage can contribute to hypertension by promoting vascular smooth muscle vasoconstriction and renal tubule sodium reabsorption; hence this enzyme is generally known as angiotensin-converting enzyme (ACE) (Corvol et al., 2004). On the other hand, ACE is also known as a relatively nonspecific peptidase that can hydrolyze a wide range of small peptides *in vitro* (Skidgel et al., 1984; Skidgel and Erdös, 1985; Kase et al., 1986; Dubreuil et al., 1989). At present, *in vivo* ACE activity has already been shown to play roles in many different physiological processes in several different tissues, including the lungs, small intestine, choroid plexus and lymphocytes (Azizi et al., 1996, 1997). The enzyme exists in two distinct membrane-bound isoforms, a double-domain somatic form (sACE; 180 kDa) and a single-domain testicular form (tACE; 110 kDa), both

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of which are transcribed from the same gene (Kumar et al., 1989; Lattion et al., 1989; Howard et al., 1990).

Studies in a range of invertebrates (e.g., arthropods and annelids) and even in some bacteria have found several soluble ACE-related carboxypeptidase activities (Rawlings et al., 2008; Rivière, 2009). The insect ACE-related protein sequences predicted from the cDNA of *Drosophila melanogaster* (Cornell et al., 1995), *Haematobia irritans exigua* (Wijffels et al., 1996), *Bombyx mori* (Quan et al., 2001), *Spodoptera littoralis* (Lemeire et al., 2008) and *Acyrthosiphon pisum* (Wang et al., 2015) have similarities to tACE, since they possess only one active site and one zinc-binding domain, and encode a secreted protein with no recognizable C-terminal hydrophobic membrane anchor sequence. Insects have an open circulatory system and do not have an RAS. Therefore, it is thought that one or more conserved "non-RAS" substrates for insect ACE play important roles in vital physiologic processes, including reproduction and development. Studies on the localization of the enzyme in different developmental stages and tissues have often led to speculations about new physiological functions. For example, it was expected that the ACE that exhibits high-level activity in the gut tissues of different larval fly species functions as a processing enzyme for gastrointestinal hormones (Wijffels et al., 1997). The localization of ACE in the neuropil regions and neurosecretory cells from a number of insect species suggests that a possible role of ACE is the metabolic inactivation of peptide neurotransmitters and/or the biosynthesis of neuropeptides (Schoofs et al., 1998). The ACE homologs in the *A. pisum* salivary glands act as effector proteins to modulate insect–plant interactions (Wang et al., 2015). In addition, ACE-like protein is abundantly present in the reproductive tissues of different insect species (Wijffels et al., 1996; Loeb et al., 1998; Schoofs et al., 1998; Isaac et al., 1999) and may play a role in their reproduction. In the tomato moth, *Lacanobia oleracea* (Lepidoptera), the male accessory gland ACE-like activity is lost after mating, presumably by a transfer to the female in the seminal fluid, suggesting a role for the peptide-processing enzyme in seminal fluid (Ekbote et al., 2003a). However, no definitive conclusions have been reached in regard to the importance of reproductive gland ACE-related proteins for male fertility.

In the present article, to address the major physiological functions of ACE in the silkworm, we use captopril, a strong and specific inhibitor of ACE, to examine the effects of the suppression of ACE-like activity on the activation of sperm motility *in vitro*. In addition, we report the transfer of ACE-like activity from the *B. mori* male seminal vesicles to the female bursa (b.) copulatrix during the mating process. Finally, we clone the ACE isoform occurring in the seminal plasma and show that it is inhibited by captopril. In the present article, to address the major physiological function of ACE in the silkworm, we use a strong and specific inhibitor of ACE, captopril to examine the effects of the suppression of ACE-like activity on the activation of sperm motility *in vitro*. In addition, we report the transfer of ACE-like activity from the *B. mori* male seminal vesicles to the female bursa (b.) copulatrix during the mating process. Finally, the ACE isoform occurring in the seminal plasma was cloned and showed to be inhibited by captopril.

2. Materials and methods

2.1. Chemicals

As a selective inhibitor for ACE activity, we used captopril ((2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid) (Nacalai Tesque, Kyoto, Japan) as a solution in 5% methanol. TPCK-treated trypsin from bovine pancreas was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Insect rearing and sample collection

B. mori (a commercially available F_1 hybrid, Kinshu × Showa) were cultured at 25 ± 1 °C under complete aseptic conditions as described previously (Nagaoka et al., 2011). Males and females were separated at the pupal stage, kept individually in plastic dishes, and allowed to develop further at 25 ± 1 °C. Adult tissues were dissected in ice-cold 0.75% NaCl solution under a dissecting microscope. The reproductive systems from virgin 1-day-old male insects were separated into the testes, ductus (d.) deferens, ampulla ductus deferens (d. d.), g. pellucida, g. lacteola, v. seminalis, g. spermatophorae (plus alba) and g. prostatica (Nagaoka et al., 2011). The b. copulatrix containing the spermatophore was excised from 0-day-old females immediately after the start of mating with 1-day-old males. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C for each experiment.

Separation of sperm and seminal plasm was fundamentally performed in accordance with Karr and Walters (2015). Seminal fluids containing apyrene and eupyrene sperm from v. seminalis (80 µl) were placed into 100 mm plastic petri dishes containing 720 µl of phosphate-buffered saline (PBS) and dispersed using a P200 pipettor fitted with a siliconized wide bore tip. The dispersed contents were deposited into 1.5 ml microcentrifuge tubes and centrifuged at 15,000 × *g* for 2 min at 15 °C. The supernatant solutions were saved as seminal plasma fractions. Following supernatant removal, the resulting pellets were suspended in 1 ml PBS. This process was repeated 20 times and provided the sperm fractions.

2.3. Sperm assays

The effects of captopril on the induction of apyrene motility and on the dissociation of eupyrene bundles were assayed essentially by the method of Osanai et al. (1989). Nonmotile sperm were teased away from two isolated v. seminalis and incubated in 200 µl of ice-cold silkworm Ringer solution (Narahashi, 1963). Volumes of 20 µl of sperm suspension were each mixed with 1 µl of a solution of the inhibitor at various concentrations on a slide glass and pre-incubated at room temperature for 2 min. Then 2 µl of a homogenate of g. prostatica (1 gland in 50 µl of Ringer solution) or trypsin (100 µg/mL) was added to the mixture in the continued presence of the inhibitor. The sperm were observed at 100 × magnification under a phase-contrast microscope for 10 min. To measure sperm velocity, 10 fields were randomly selected at the microscope; each field contained at least five eupyrene bundles. Images were recorded using a Leica MC120HD camera (Leica Microsystems, Wetzlar, Germany). Treated sperm were quantified using a rank scale (Table 1). It should be noted that the homogenate of g.

Table 1
Motility and dissociation level ranking.

Rank	Motility level of apyrene sperm	Dissociation level of eupyrene sperm bundle
0	Non-motile	Non-dissociate (compacted)
1	< 10% of sperm exhibiting partial motility including twitching sperm	< 50% of sperm bundle exhibiting swelled in the head part
2	Between 10% and 75% of sperm exhibiting partial motility including twitching sperm or slow waves	Between 50% and 75% of sperm bundle exhibiting partial and/or complete dissociationof a whole bundle
3	> 75% of sperm exhibiting full motility	> 75% of sperm bundle exhibiting complete dissociation of a whole bundle